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CHANGES IN THE ACTIVITIES OF NUCLEIC ACID-DEGRADING ENZYMES DURING THE LIFE CYCLE OF *Saccharomyces cerevisiae*

by

Donald Ivan Van Ryk

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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DEPARTMENT OF MICROBIOLOGY

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FALL, 1984

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled **CHANGES IN THE ACTIVITIES OF NUCLEIC ACID-DEGRADING ENZYMES DURING THE LIFE CYCLE OF** *Saccharomyces cerevisiae* submitted by Donald Ivan Van Ryk in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE in MICROBIOLOGY.

DEDICATION

To my Mother and Father, who

always wanted the best for me.

ABSTRACT

The changes in activity of nucleic acid-degrading enzymes throughout the life cycle of *Saccharomyces cerevisiae* SK-1 were investigated in an effort to clarify their physiological functions. Three enzymes were studied; a Mg^{2+}-independent endonuclease, a Mg^{2+}-dependent endonuclease and a Mg^{2+}-dependent 5'-exonuclease. Since all three nucleases were most active with poly(A) as substrate, it was necessary to establish methods to specifically assay their activities in crude cell extracts.

As the cells entered stationary phase and sporulation, the specific activity of the Mg^{2+}-independent endonuclease increased about 4- and 6-fold, respectively. The specific activity of the Mg^{2+}-dependent 5'-exonuclease increased 2- and 7-fold, respectively, while the specific activity of the Mg^{2+}-dependent endonuclease decreased to about 50% of its original value under these conditions in both the haploid and diploid strains. During the first few hours of sporulation, the specific activity of the Mg^{2+}-dependent endonuclease increased about 2-fold in the diploid, but not the haploid strain. When the haploid cells were transfered from sporulation to germination conditions, the activities of the Mg^{2+}-independent endonuclease and Mg^{2+}-dependent 5'-exonuclease decreased while the Mg^{2+}-dependent endonuclease activity increased. From these changes in activity and the general characteristics of the enzymes, it was deduced that the Mg^{2+}-independent endonuclease may be

involved in the degradation of rRNA while the Mg^{2+}-dependent 5'-exonuclease activity probably plays a role in the degradation of mRNA. The Mg^{2+}-dependent endonuclease may be involved in the recombination and/or repair of DNA, although a role in RNA metabolism cannot be ruled out. By inhibiting protein synthesis with cycloheximide it was found that the three enzymes were very stable *in vivo* under most conditions. It is likely that any increases in specific activity were due to *de novo* synthesis while any decreases were due to degradation of the enzymes.

An activity stain for the Mg^{2+}-independent endonuclease on polyacrylamide gels was used to investigate its electrophoretic properties. Two bands of activity were seen when the nuclease was isolated from stationary phase cells while only the slower migrating activity (band 1) was seen in significant amounts in exponential phase cell extracts. Attempts to isolate the two components by column chromatography using Sephadex G-100 superfine and DEAE-Trisacryl, both in the presence of SDS were unsuccessful. This suggests that band 1 (MW >>100,000 d) is an aggregate of band 2 (MW 50,000-68,000 d). A third band which migrated slightly faster than band 2 was observed in some cases. Whether this represented a separate activity, a different form of band 2 or an artifact of electrophoresis could not be determined.

The relative activity of the Mg^{2+}-independent endonuclease with poly(C), poly(A), RNA, ssDNA and dsDNA as

substrate was 139.4, 100, 47.5, 0.2 and 0.3, respectively. Poly(A) was degraded to 3'-phosphate terminated oligonucleotides. The pH optimum was 7.0 and 6.5 for the enzyme isolated from exponential- and stationary phase cells, respectively. Whether this difference is due to the presence of a unique enzyme in stationary phase or to the apparent ability of the nuclease to aggregate remains to be determined. The Mg^{2+}-independent endonuclease had a temperature optimum of 55°C but was rapidly inactivated at 60°C. However, in the presence of SDS, full activity could be recovered after heating at temperatures up to 95°C if the SDS was later replaced with Triton X-100.

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LIST OF ABBREVIATIONS

A.....absorbance

α.....alpha

β.....beta

BSA.....bovine serum albumin

Ci.....currie

DEAE.....diethylaminoethyl-

DNA.....deoxyribonucleic acid

DNase.....deoxyribonuclease

EDTA.....ethylenediaminetetracetic acid

γ.....gamma

g.....gram

L.....liter

m.....milli-

M.....molar

mol.....mole

MOPS.....morpholinopropane sulfonic acid

MPa.....megapascals

mRNA.....messenger ribonucleic acid

mt-DNA.....mitochondrial deoxyribonucleic acid

OD.....optical density

OVA.....ovalbumin

PCA.....perchloric acid

PIPES.....piperazine-N,N'bis(2-ethane sulfonic acid)

PMSF.....phenylmethylsulfonyl fluoride

Poly(A).....polyadenylic acid

Poly(C).....polycytidylic acid

POPOP.....1,4-bis-[2-(5-phenyloxazolyl)]-benzene

PPO.....-2,5-diphenyloxazole

RNA.....ribonucleic acid

RNase.....ribonuclease

rRNA.....ribosomal ribonucleic acid

SDS.....sodium dodecyl sulfate

TCA.....trichloroacetic acid

TEMED.....N,N,N',N'-tetramethylethylenediamine

Tris.....tris(hydroxymethyl)aminomethane

tRNA.....transfer ribonucleic acid

μ.....micro

xg.....times the force of gravity

I. INTRODUCTION

A. Life Cycle of *Saccharomyces cerevisiae*

The yeast *Saccharomyces cerevisiae* has been studied extensively since it is economically important and can be used as a model system in cell biology and cell physiology. The life cycle, as illustrated in Fig 1, is of particular interest since it involves both mitotic and meiotic phases and, therefore, developmental processes of eukaryotes can be studied using this organism.

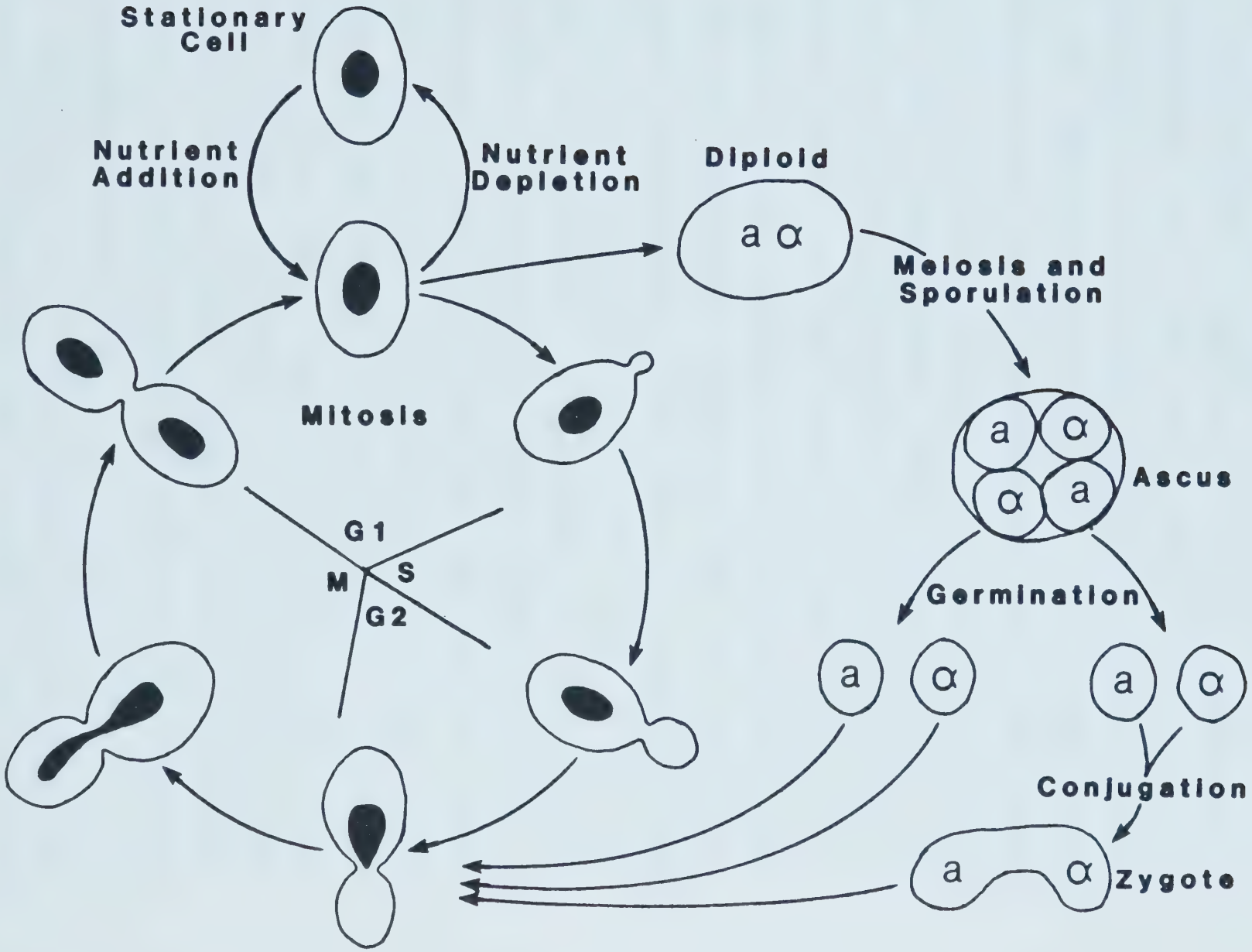
The landmarks of the mitotic cycle have been well defined. DNA synthesis occurs in the S period shortly after bud emergence and this is followed by additional growth in the G2 period. Nuclear migration and division occur in the M period which is followed by the G1 phase where the cells divide. Under conditions of balanced growth, all of the cell's macromolecular components are doubled once each cycle (Hartwell, 1974). Growth does not appear to be dependent on the completion of any landmark since cells arrested at these positions grow in size after the time of arrest (Hartwell, 1974).

When yeast are placed under conditions of nutrient deprivation, they enter stationary phase and cell division is arrested in the G1 phase prior to DNA synthesis (Williamson and Scopes, 1962). While some stationary phase cells are of normal size, others are very small (Williamson and Scopes, 1961) and, on the average, cells contain less

FIGURE 1

Life Cycle of *Saccharomyces cerevisiae*

Schematic diagram of the life cycle of *S. cerevisiae* showing both mitotic and meiotic phases as well as the germination of spores. G1, S, G2 and M represent the various stages in the mitotic cell cycle. The unlabelled cells may be either haploid or diploid while "a" and "α" represent the mating type alleles.
Modified from Hartwell (1974)


Stationary Cell
Nutrient Addition
Nutrient Depletion
Diploid
a α
Meiosis and Sporulation
Mitosis
G1
S
M
G2
a
α
Ascus
Germination
Conjugation
Zygote

mass, protein and RNA than the average growing cells (Polakis and Bartley, 1966).

When diploid *S. cerevisiae*, which are heterozygous at the mating type allele, are inoculated into a medium containing a non-fermentable carbon source, such as acetate, in the absence of a nitrogen source, they undergo meiosis and sporulation (Esposito and Klapholz, 1981). The meiotic process is initiated during the G1 interval of the mitotic cell cycle (Croes, 1966). Cells at any of the other intervals must complete the cell cycle first before entering meiosis since the first meiotic nuclear division is reductional while mitotic nuclear division is not (Croes, 1966). The landmarks of meiosis include DNA replication, genetic recombination, first and second nuclear divisions and spore wall formation (Moens and Rapport, 1971). The rates of synthesis and breakdown of macromolecules, primarily RNA and protein, increase considerably as soon as sporulation starts and this continues until asci appear (Hopper *et al*, 1974). Cytokinesis and cell division during meiosis result from the growth of new cell wall and cell membrane within the cytoplasm of the mother cell, rather than by septum formation as in mitotically growing yeast (Moens, 1971). The newly developed spores are contained within a modified cell called an ascus and are maintained in a semi-dormant state.

When the spores are returned to a growth medium, this dormancy is broken and they germinate. The resulting

vegetative cells are haploid and as such may continue into the mitotic growth cycle, or cells of opposite mating type may undergo conjugation to form a diploid zygote. The conjugation process involves several distinct steps. Each haploid cell type constitutively produces and secretes specific pheromones called a- or α-mating factors which trigger biochemical changes in the cells of opposite mating type (Thorner, 1981). These changes include cell surface alterations to enhance specific intercellular adhesion and synchronization of the growth stages of the mating partners by arresting the cell cycle in the G1 phase. Once these first steps have been completed, the cell wall and plasma membrane are removed at the point of contact by autolytic activities. Zygote formation is complete when the haploid nuclei fuse to form a single diploid nucleus (Thorner, 1981). These cells may then continue the mitotic cell cycle and under the appropriate conditions can again sporulate.

Complex changes in morphology and composition occur as the cells proceed through the cell cycle. As a result, it is expected that changes in enzyme activities reflecting different rates of biosynthesis and degradation of macromolecules will also be observed.

B. Nucleic Acid Metabolism

During mitotic growth of *S. cerevisiae*, total cell mass, protein and RNA accumulate continuously throughout the cell cycle (Tauro and Halvorson, 1966; Sebastian *et al*,

1971). Yeast cells contain 50 to 100 times more RNA than DNA (Mounolou, 1971) and over 80% of the RNA is ribosomal RNA composed of 4 species; 5s, 5.8s, 18s and 26s (Kozak, 1983). The 5.8s, 18s and 26s species are obtained from a larger 35s precursor which is in turn derived from a 45s transcript (Perry, 1981).

About 1-2% of the total RNA in yeast is made up of messenger RNA (Tonnensen and Friesen, 1973). Messenger RNA is the least stable class of RNA and is degraded about 6 times faster than rRNA, following the kinetics of exponential decay (Elliott and McLaughlin, 1979; Chia and McLaughlin, 1979). Several studies have determined that the average half-life of total mRNA is about 20 min (Tonnensen and Friesen, 1973; Chia and McLaughlin, 1979; Koch and Friesen, 1979), but the half-life of an individual mRNA ranges widely from 3.5 min to more than 70 min (Koch and Friesen, 1979). The reason for this difference in stability is not clear but some suggestions have been advanced to account for it. One explanation involves the observation that there are two classes of mRNA with different stabilities. One class has 5'-termini of m^7GpppA and the other has 5'-termini of m^7GpppG. These classes exist at a ratio of approximately 3:1, respectively, and the RNAs with the m^7GpppG cap are degraded 4 to 5 times more rapidly than those capped with m^7GpppA (Scripati, *et al*, 1976). Another explanation for the variation in the mRNA stability may be the differences in the length of the polyadenylic acid tails

added post-transcriptionally to the 3'-OH end. Again, there are different classes of mRNA, one having a poly(A) tail about 90 nucleotides long, and one having a poly(A) tail only 20-30 nucleotides long and a third class having no poly(A) tail (Sogin and Saunders, 1980). The loss of the poly(A) tail may be a mechanism whereby mRNAs which normally have a long half-life can be degraded more rapidly, since de-adenylated mRNA has been found to be less stable in *Xenopus* oocytes (Huez *et al*, 1974).

As the cells enter stationary phase the rates of degradation of protein and RNA increase (Hopper *et al*, 1974). Stationary phase cells only have half the poly(A) content of exponential phase cells, and this is partly due to a decreased proportion of mRNA with the long poly(A) tail compared to the shorter one and partly due to the fact that fewer mRNAs have any poly(A). Since the class of mRNA with the 20-30 nucleotide poly(A) tail predominates in stationary phase cells, the variation in poly(A) tail length may partly account for the observed increase in RNA degradation as well as differences in individual mRNA half-lives (Sogin and Saunders, 1980).

Sporulation is accompanied by extensive synthesis and degradation of proteins and RNA (Hopper *et al*, 1974).The maximum rate of RNA synthesis occurs at about 5-6 hrs after the initiation of sporulation (Hopper *et al*, 1974) but the breakdown of RNA begins immediately and results in an overall decrease in the total RNA content of 50% by the

completion of spore formation (Croes, 1967a).

A new 20s rRNA species which has not been detected in non-sporulating cells is synthesized during sporulation in yeast (Kadowaki and Halvorson, 1971). The relative content of this RNA to the total RNA content increases linearly early in sporulation. However, the function of this species of rRNA is not known.

The decrease in total RNA content of the cells which occurs under conditions of nutrient deprivation or sporulation is due mostly to the degradation of rRNA (Croes, 1967b). The number of ribosomes in polysomes decreases 3-fold during sporulation (Kraig and Haber, 1980) and this may result in a decrease in rRNA stability, since rRNA in intact polysomes is not affected by nuclease levels which readily degrade the ribosomal subunits (Utsunomiya and Roth, 1966).

Transfer RNA, which accounts for about 17% of the RNA of vegetative cells, is reasonably stable and is present in approximately the same ratio to mRNA throughout the life cycle (Esposito *et al*, 1969).

The breakdown of total RNA observed during stationary phase and sporulation is due to an increase in the activities of nucleolytic enzymes at these times (Clare and Oliver, 1979). The cells presumably depend upon the degradation of existing RNA for their supply of nucleotides since strains which cannot increase their total nuclease activities during sporulation are defective in ascus

formation (Tsuboi, 1976).

About 5-20% of the total yeast DNA is located in the mitochondria and the remainder is found in the nucleus (Hartwell, 1974). Several studies have described nucleic acid-degrading enzymes which hydrolyse either ssDNA or dsDNA or both. As will be described in the following section, some of these may function in the recombination and repair of DNA.

C. Nucleic Acid-Degrading Enzymes

A number of nucleic acid-degrading enzymes isolated from *S. cerevisiae* and related organisms have been described. It has been suggested that nuclease activities in various organisms are involved in the degradation of mRNA (Lehman, 1963), destruction of ribosomes during sporulation (Farkas and Marks, 1965), control of protein synthesis by destruction of specific tRNAs (Stent, 1964; Nishimura and Novelli, 1964), and recombination of DNA (Resnick *et al*, submitted for publication). While many suggestions have been advanced concerning the functional roles of nucleic acid degrading enzymes of yeast, little has been confirmed.

Nucleic acid-degrading enzymes also are involved in processing of newly synthesized RNA. All of the classes of RNA are transcribed as larger primary transcripts which are processed into the functional forms of RNA in the nucleus. This processing includes nucleolytic cleavages, ligations, terminal additions and nucleoside modifications (Perry,

1981). In yeast, the nucleases which are responsible for the cleavage of these primary transcripts have not been identified, but the enzymes which have been described could play a role in the degradation of the large sequences which are discarded during RNA processing.

The nucleases which have been described can be divided into two main classes. One class is active in the presence of EDTA and have been termed the Mg^{2+}-independent endonucleases. The enzymes in the other class require the presence of Mg^{2+} or related ions for activity and are called Mg^{2+}-dependent nucleases. This class includes a membrane bound endonuclease and a Mg^{2+}-dependent 5'-exonuclease associated with the ribosomes. One nuclear deoxyribonuclease has also been described (Bryant and Haynes, 1977) but little is known about it and its function has not been determined.

The major characteristics of the Mg^{2+}-independent endonucleases isolated from *S. cerevisiae* in several different investigations are listed in Table 1. For the most part, their characteristics are very similar, indicating that these may be slightly different forms of the same enzyme. The differences observed may, in some cases, be due to variations in the assay conditions used.

All of these enzymes are endonucleases which are active in the presence of high concentrations of EDTA and are relatively heat stable. In general, they are most active with RNA, poly(A) or poly(U) as substrate and are virtually inactive with either native or heat denatured DNA.

TABLE 1

A Summary of the Properties of the Mg^{2+}-independent Endonucleases Isolated from *Saccharomyces cerevisiae*

Subcellular Distribution	Substrates	Products	Ion Requirement	Inhibitors	pH Optimum	Proposed Function	Reference
Cytoplasm	RNA	Oligonucleotide -3'-P	None	Zn^{2+}	7.5	-	Ohtaka *et al*, 1963
Ribosomes	Poly(A) Poly(U) RNA	Nucleoside -3'-P	None	None	7.0	Degradation of mRNA	Nakao *et al*, 1968
40s Ribosomal Subunit	rRNA tRNA Poly(A) Poly(U) Poly(C)	Oligonucleotide -3'-P	None	Zn^{2+} Mn^{2+}	5.0-6.5	Degradation of Ribosomes	Schulz-Harder *et al*, 1979
Cytoplasm	RNA	Nucleoside -3'-P	Na^{2+} or K^{+}	Cu^{2+} Fe^{2+}	6.0	-	Shetty *et al*, 1980
Chromatin	ssRNA Poly(C) Poly(U)	Oligonucleotide -5'-P	None	50% by 1 m*M* ATP or CTP	-	Degradation of RNA after Heat Shock	Belhadj *et al*, 1982

Several functions have been proposed for these enzymes. Nakao *et al* (1968) suggested that the enzyme they studied is involved in the degradation of mRNA, although it may also degrade other classes of RNA. Schulz-Harder (1983) describes a nuclease which is newly synthesized following heat shock treatment and suggests that it may be involved in the degradation of ribosomes. An acidic RNase with characteristics which are similar to the Mg^{2+}-independent endonuclease of *S. cerevisiae* was isolated from *Candida lipolytica* by Imada *et al* (1975) and they also propose a role in RNA degradation following heat shock treatment for their enzyme. However, unlike the enzyme described by Schulz-Harder, 50-75% of this nuclease is found in an inactive form bound to a thermolabile inhibitor. Shetty *et al* (1980) do not propose a function for the enzyme they investigated, but it may have an analogous function to those described by Schulz-Harder *et al* and Imada *et al* since they all have similar characteristics. Belhadj *et al* (1982) also conclude that the RNase they describe is involved in the digestion of RNA following heat treatment. However, it may also function in the degradation of RNA under conditions of nutrient limitation since it is activated by low concentrations of ATP and CTP but inhibited up to 50% by high concentrations of these nucleotides. This enzyme differs from the other Mg^{2+}-independent endonucleases in that it produces 5'-phosphate terminated oligonucleotides.

The Mg^{2+}-dependent endonuclease associated with the inner mitochondrial membrane is a major nucleic acid-degrading enzyme in yeast. It now appears that the same enzyme may have been studied in a number of independent investigations. Several of the major characteristics of the enzymes reported in these investigations are listed in Table 2.

There are significant differences in the characteristics of some of these enzymes but variations in the assay conditions used and the extent of purification in different investigations make it difficult to compare the nucleases. However, it seems likely that many of the reports describe the same enzyme. Most investigators describe this enzyme as a deoxyribonuclease, but do not report the activities with other substrates such as RNA. Von Tigerstrom (1982) found that it is most active with poly(A) as substrate and the relative rates of hydrolysis of poly(A), ssDNA, RNA, dsDNA and poly(C) are 100, 31, 19, 2.1 and <0.2, respectively. Jacquemin-Sablon *et al* (1979) state that their preparation is free of RNase activity as measured with poly(C), but this nuclease is virtually inactive with this substrate. However, in general the enzyme appears to be more active with ssDNA than RNA as substrate so it is quite possible that it is a single strand-specific DNase *in vivo*.

A Mg^{2+}-dependent endonuclease isolated from the mitochondria of *Neurospora crassa* has also been reported (Linn and Lehman, 1966; Martin and Wagner, 1975; Chow and

Table 2

A Summary of the Properties of the Mg^{2+}-dependent Endonucleases Isolated from *Saccharomyces cerevisiae*

Subcellular Distribution	Substrates	Products	Ion Requirement	Inhibitors	pH Optimum	Proposed Function	Reference
-	ssDNA	Oligonucleotide -5'-P	Mg^{2+} or Mn^{2+}	EDTA	6.0	Recombination	Pinon, 1970
Mitochondrial Membrane	ssDNA	-	Mg^{2+} or Mn^{2+}	EDTA	6.2-7.8	Regulation of mt-DNA Replication	Jacquemin-Sablon *et al*, 1979
Mitochondria	dsDNA	Mono-, Di- and Trinucleotide -5'-P	Mg^{2+} or Mn^{2+}	EDTA	7.0	Initial Step in Recombination	Morosoli and Lusena, 1980
Mitochondria	ssDNA	-	Mg^{2+} or Mn^{2+}	EDTA	7.6	-	Rosamond, 1981
-	Apurinic DNA	-	Mg^{2+} or Mn^{2+}	EDTA	6.0-8.0	Repair of Damaged DNA	Akhmedov *et al*, 1982
Mitochondria	Apurinic DNA	-	Mg^{2+}	EDTA Zn^{2+}	7.0-7.5	Repair of mt-DNA	Foury, 1982
Mitochondrial Membrane	Poly(A): ssDNA 100:31	Small Oligonucleotide -5'-P	Mg^{2+} or Mn^{2+}	EDTA	6.5-7.0	-	von Tigerstrom, 1982

Fraser, 1983). In all of these studies the enzyme has characteristics which are very similar to those reported for the *S. cerevisiae* Mg^{2+}-dependent endonuclease suggesting a possible similarity in function.

The enzyme described by Chow and Fraser (1983) acts as an endonuclease with ssDNA and RNA and as an exonuclease with dsDNA as substrate. However, if it is isolated in the absence of protease inhibitors, a nuclease similar to the one described by Linn and Lehman (1966) appears. Therefore, it is possible that the enzymes described by all of these investigators represent slightly different forms of the same activity.

Von Tigerstrom and Stelmaschuck (submitted for publication) compared the properties of the Mg^{2+}-dependent endonuclease isolated from *S. cerevisiae* and *N. crassa*. They found that while these activities were not closely related antigenically, the similarities in substrate specificity, mode of attack and intracellular location indicated that the enzymes have the same function *in vivo*.

Several suggestions have been made concerning the functions of these enzymes. These include the regulation of mitochondrial DNA replication (Jacquemin-Sablon *et al*, 1979), the repair of mt-DNA (Foury, 1982; Fraser and Cohen, 1983) and a role in recombination, particularly during differentiation, (Pinon, 1970; Morosoli and Lusena, 1980; Chow and Fraser, 1983). However, little evidence has been obtained to support these proposals.

The other Mg^{2+}-dependent nuclease is an exonuclease described by Stevens (1978, 1979, 1980) which degrades RNA in a 5'-->3' direction. It has a broad pH optimum around 8.0-8.5 and is completely inhibited by EDTA. Ribosomal RNA is hydrolysed at nearly the same rate as poly(A) yielding 5'-mononucleotides as end products. The enzyme requires 5'-phosphates and 5'-capped mRNAs are hydrolysed slowly, if at all. Stevens suggests that its function *in vivo* may be to degrade mRNA and this nuclease, in conjunction with an enzyme that removes 5'-caps, may be very important in determining the functional half-lives of mRNA. In addition, since the direction of degradation is the same as the direction of translation, incomplete protein synthesis would not result from the translation of partially degraded RNA.

Villadsen *et al* (1982) also describe an exonuclease which acts in a 5'-->3' direction and it is very similar to the enzyme described by Stevens. They state that it degrades ssDNA to yield 5'-deoxyribonucleotides and that it also digests poly(A), 16s and 23s RNA, but they do not indicate how active the enzyme is with these last three substrates. Therefore, it seems likely that Stevens and Villadsen *et al* have described the same or similar activities.

In summary, total RNA concentrations increase continuously throughout the mitotic cell cycle (Elliott and McLaughlin, 1979), but under conditions of nutritional deprivation, the RNA content of the cells decreases by as much as 50% (Croes, 1967a). Nucleolytic enzymes play a very

important role in the metabolism of nucleic acids. Several such enzymes have been investigated and can be placed in two broad classes. These are designated Mg^{2+}-independent nucleases and Mg^{2+}-dependent nucleases. The second class can be further broken down to include a membrane-bound endonuclease and a Mg^{2+}-dependent 5'-exonuclease. These enzymes must act specifically and in a highly controlled manner, but, as yet, little is known about their control or function.

The purpose of this project was to follow the changes in the activity of these nucleases throughout the life cycle of *S. cerevisiae*. In this way it was hoped that a better understanding of the specific *in vivo* roles of these enzymes could be gained. Since they are most active with the same substrate, methods were devised to distinguish between the different nucleases. Finally, the Mg^{2+}-independent endonuclease was studied to characterize it more completely.

II. MATERIALS AND METHODS

A. Materials

Triton X-100, heparin agarose, cycloheximide, poly(A), poly(C) and calf thymus DNA were all obtained from the Sigma Chemical Co. The DNA was used as the source of double-stranded DNA (dsDNA). The single-stranded DNA (ssDNA) was prepared by heating the dsDNA at 100°C for 10 min and then cooling it rapidly. L-leucine-^{14}C (uniformly labelled) (308 μCi/μmol) was purchased from New England Nuclear and Poly(8-^{3}H)-Adenylic acid (574μCi/μmol) was obtained from Amersham. High molecular weight RNA and Zwittergent 3-14 were purchased from Calbiochem. Sephadex G-100 superfine was obtained from Pharmacia Fine Chemicals while Zymolyase 60,000 was obtained from the Kirin Brewery Co. Ltd. N,N'-methylenebisacrylamide and acrylamide were both purchased from Eastman Kodak. All other materials and chemicals were purchased commercially.

The media and solutions used in this investigation are as follows:

Yeast Extract-Glucose Broth (pH 4.5)

Yeast Extract	20 g
Glucose	10 g
Water	to 1 L

Presporulation Medium (Roth and Halvorson, 1969)

Yeast Nitrogen Base without Amino Acids	6.7 g
Yeast Extract	1 g
Potassium Acetate	10 g
Potassium Phthalate (0.05 *M*, pH 5.0)	to 1 L

Sporulation Medium (pH 6.5)

Potassium Acetate	10 g
MOPS	41.86 g
Water	to 1 L

Brays Scintillation Fluid

Naphthalene	60 g
PPO	4 g
POPOP	0.2 g
Absolute Methanol	100 mL
Ethylene Glycol	20 mL
Para-dioxane	to 1 L

Buffer 1: 50 m*M* Tris-Cl, 10 m*M* KCl, 5 m*M* $MgCl_2$, pH 7.0

Buffer 1a: 50 m*M* Tris-Cl, 2 *M* KCl, 5 m*M* $MgCl_2$, pH 7.0

Buffer 2: 50 m*M* Tris-Cl, 75 m*M* KCl, 5 m*M* $MgCl_2$, 25 m*M* potassium phosphate, 0.2% Zwittergent 3-14, pH 6.0

Buffer 3: 50 m*M* Tris-Cl, *M* KCl, 5 m*M* $MgCl_2$, 25 m*M* potassium phosphate, 0.2% Zwittergent 3-14, pH 6.0

B. Methods

Growth and Maintenance of the Organism

Saccharomyces cerevisiae SK-1, obtained from the collection of Dr. R. von Tigerstrom, and a haploid strain derived from this organism were used for all experiments. The cells were maintained on phytone yeast extract agar at 4°C. To obtain cells for the experiments, the organisms were grown routinely in yeast extract-glucose broth. Cultures grown to stationary phase (15-20 OD_{600}) and stored at 4°C were used as inoculum.

Unless stated otherwise, cultures were incubated at 30°C with shaking at 250 rpm. Centrifugations were carried out at 1600xg for 5 min at 4°C and washing of cells was performed by suspension into the indicated solutions followed by centrifugation.

Sporulation of *S. cerevisiae* SK-1

The following describes a typical sporulation experiment. All manipulations were carried out aseptically using sterilized solutions and containers. A 16 hr culture with an OD_{600} of 15-20 was harvested by centrifugation and washed twice with cold water. The cells were suspended in presporulation medium to obtain $5x10^6$ cells/mL and incubated without shaking at 30°C for 24-48 hrs. These cells were collected by centrifugation, washed once with cold water, resuspended to a density of $5x10^7$ cells/mL in sporulation medium and incubated for 24 hrs. The formation of asci was followed under the phase contrast microscope.

Germination of *S. cerevisiae* SK-1 Spores

S. cerevisiae was sporulated as described above. The asci were harvested by centrifugation and washed once in cold water. They were then suspended in 1/10 of the volume of buffer 1 and incubated with 0.2 mg/mL Zymolyase 60,000 and 2.5 μL/mL β-mercaptoethanol for 30 min at 30°C to weaken the ascus wall. The asci were then washed twice, resuspended in the original volume of buffer 1 and passed through a

French pressure cell at 20.7 MPa. The free ascospores were centrifuged at 1600xg for 10 min at 4°C and incubated in yeast extract-glucose broth. Outgrowth was followed under the phase contrast microscope by counting about 200 cells and determining the proportion of refractile ascospores to non-refractile vegetative cells.

Obtaining a Haploid Strain of *S. cerevisiae* SK-1

To obtain a haploid strain from *S. cerevisiae* SK-1, asci were harvested by centrifugation, washed once with cold water, and then suspended in buffer 1 to a final density of $5x10^5$ asci/mL. The cells were incubated with 0.4 mg/mL Zymolyase 60,000 for 10 min at 30°C and the spores were released from the ascus at 0-4°C by three 1 min bursts with a Bronwill Biosonik sonicator using maximum power. This was followed with the phase contrast microscope. After washing the spores twice with cold water, they were resuspended in water to $5x10^5$ spores/mL and plated onto phytone yeast extract agar to obtain isolated colonies. After incubation at 30°C, several of the smaller colonies were selected, grown in yeast extract-glucose broth and placed under sporulation conditions. Cultures that did not sporulate were chosen as possible haploid isolates. To confirm that these cells were haploid, their DNA content was determined by the method of Stewart (1975) as described under analytical methods. The diploid strain was expected to contain about 5 μg DNA/10^8 cells (Fasman, 1970). The haploid isolate

contained about 2.6 μg DNA/10^8 cells while the diploid strain contained 4.8 μg DNA/10^8 cells.

Preparation of Cell Extracts

Two methods of preparing cell extracts were investigated. These were sphaeroplast formation and passing whole cells through a French pressure cell.

Preparation of Sphaeroplasts

The cells were harvested by centrifugation and washed in the cold with water followed by 1 *M* sorbitol, 0.1 *M* potassium phosphate, pH 7.5 (sphaeroplasting buffer). The cells were then suspended in sphaeroplasting buffer to 0.15 g (wet weight) per mL. After adding 2.5 μL/mL β-mercaptoethanol and 0.4 mg/mL Zymolyase 60,000 the cells were incubated at 30°C with occasional gentle agitation. Sphaeroplast formation was observed by phase contrast microscopy and by determining the decrease in the OD_{600} after aliquots were diluted with water. When sphaeroplast formation was complete, they were washed twice by centrifugation at 800xg for 10 min at 4°C and gentle resuspension in sphaeroplasting buffer.

Preparation of French Pressure Cell Lysate

Cells harvested by centrifugation were washed twice with cold water and once with cold buffer 1. They were suspended in cold buffer 1 to obtain 1.5 g cells (wet weight) per mL and passed twice through a French pressure cell at 103 MPa. From cell counts using the haemacytometer

it was judged that this resulted in the breakage of more than 90% of the cells.

Separation of Nuclease Activities

The cells were usually disrupted with a French pressure cell. The Mg^{2+}-independent endonuclease and the 5'-exonuclease were solubilized by making the cell extracts 2 *M* KCl by addition of solid KCl and shaking for at least 30 min at 0-4°C, followed by centrifugation at 100,000xg for 60 min. The supernatants were kept and the pellets were suspended to one half of the original volume in buffer 1a and they were centrifuged again. The supernatants from the first and second centrifugations containing the Mg^{2+}-independent endonuclease and Mg^{2+}-dependent 5'-exonuclease activities were pooled and the pellets were suspended to the original volume with buffer 1a. The Mg^{2+}-dependent nuclease was found in the 100,000xg pellet and was solubilized by adding Zwittergent 3-14 to 0.2%.

Heparin Agarose Chromatography

The Mg^{2+}-independent endonuclease and Mg^{2+}-dependent 5'-exonuclease were isolated by column chromatography at 4°C using heparin agarose as the support. The Mg^{2+}-dependent endonuclease was isolated on similar columns except all solutions contained 0.2% Zwittergent 3-14 and chromatography was carried out at 8-10°C. The following describes the method used for chromatography of the Mg^{2+}-dependent

endonuclease. The columns (1.2x40 cm) were packed in buffer 3, washed with at least one column volume of the same buffer, and then equilibrated with buffer 2. The enzyme source was dialysed overnight at 8-10°C against two changes of buffer 2 and then centrifuged at 20,000xg for 20 min to remove any precipitates before being applied to the column. The column was eluted with 1 column volume of buffer 2 and then with a linear salt gradient consisting of about 2 column volumes each of buffers 2 and 3. Fractions collected were assayed for nuclease activity and the protein was measured by determining the absorbance at 280 nm.

Gel Filtration

Sephadex G-100 superfine, suspended in 10 m*M* Tris-Cl, 0.1 *M* NaCl, 0.1% SDS, pH 7.5 was used to pack a 2.5x43 cm column at room temperature. It was washed with at least 1 column volume of the same buffer at a pressure of 45 cm of water. Five mL samples were applied and eluted with a flow rate of 20 mL/hr. Fractions of 2.5 mL were collected and analyzed for enzyme activity and for protein (A_{280}).

Enzyme Assays

The Mg^{2+}-independent and Mg^{2+}-dependent endonuclease activities were assayed as described by von Tigerstrom (1982). The assay for the Mg^{2+}-dependent 5'-exonuclease is based on the method described by Stevens (1978).

Total Nuclease

The total nuclease activity was measured by adding 50 μL of the enzyme source in buffer 1 with 0.2% Zwittergent 3-14 to 150 μL of 90 m*M* Tris-Cl, 25 m*M* potassium phosphate, 0.2 *M* KCl, 5 m*M* $MgCl_2$, 1 mg/mL poly(A), pH 7.0 in 400 μL microfuge tubes. This mixture was incubated at 37°C and the reaction was stopped by the addition of 100 μL 12% PCA. The tubes were incubated at 0°C for at least 20 min and then centrifuged for 1 min in a Beckman microfuge. Two hundred μL of the supernatant was diluted with 800 μL of 4% PCA and the absorbance was determined at 260 nm.

Mg^{2+}-independent Endonuclease

The Mg^{2+}-independent endonuclease activity was determined as described for the total nuclease except that the enzyme source in buffer 1 was added to an assay solution containing 90 m*M* Tris-Cl, 25 m*M* potassium phosphate, 0.2 *M* KCl, 20 m*M* EDTA, 1 mg/mL poly(A), pH 7.0.

Mg^{2+}-dependent Endonuclease

The Mg^{2+}-dependent endonuclease activity was determined by subtracting the Mg^{2+}-independent endonuclease activity from the total nuclease activity.

The units (μmol of acid-soluble nucleotides released per min) of enzyme activity were determined using ϵ=11,000 for acid-soluble products from RNA and DNA, ϵ=14,200 for acid-soluble products from poly(A), and ϵ=6,500 for acid-soluble products from poly(C) (Beard and Razzell,

1964).

Mg^{2+}-dependent 5'-exonuclease

The Mg^{2+}-dependent 5'-exonuclease activity was determined by adding 50 µL of enzyme source diluted in buffer 1 or buffer 1 with 0.2% Zwittergent 3-14 to 100 µL of 0.125 *M* Na-glycinate, 9 m*M* $MgCl_2$, 0.37 mg/mL BSA, 0.185 *M* NH_4Cl, pH 9.5. This was incubated for 5 min at 37°C in 400 µL microfuge tubes before the reaction was started by adding 50 µL of 0.2 µCi/mL ^{3}H-poly(A) (267 µCi/µmol). The reactions were stopped by adding 50 µL of 10 mg/mL BSA and 50 µL 16% PCA, 0.75% uranyl acetate. The tubes were incubated at 0°C for at least 20 min and then centrifuged for 1 min in a Beckman microfuge. Two hundred µL of the supernatant was added to 5 mL Brays scintillation fluid for the determination of acid-soluble radioactivity. The Mg^{2+}-dependent 5'-exonuclease activity was estimated by subtracting the activity obtained in the presence of 0.2% Zwittergent 3-14 from the nuclease activity obtained in the absence of the detergent.

Polyacrylamide Gel Electrophoresis of Mg^{2+}-independent Endonuclease

Polyacrylamide gel electrophoresis was carried out according to the method of Maizel (1971). The gels were cast between glass plates of 14.5 cm x 14 cm x 1.5 mm. To prepare 40 mL of 7.5% separating gel, 5 mL 3 *M* Tris-Cl pH 8.7, 10 mL acrylamide:bisacrylamide (30%:0.8%), 0.4 mL 10% SDS, 0.1 mL

TEMED and 24.4 mL water were combined at room temperature. To prepare 10 mL of spacer gel, 1.25 mL 0.5 *M* Tris-Cl pH 6.7, 1 mL acrylamide:bisacrylamide(30%:0.8%), 0.1 mL 10% SDS, 25 μL TEMED and 7.6 mL water were combined at room temperature. These solutions were de-gassed under a vacuum for at least 15 min before adding 100 μL and 50 μL of 10% ammonium persulfate, respectively. The electrode buffer was 5 m*M* Tris-glycine, 0.1% SDS, pH 8.0. Electrophoresis was carried out at a constant current of 12 mA through the spacer gel and 40 mA through the separating gel.

Samples were prepared by adding SDS to 1% and boiling for 2 min before being applied to the gels. The protein standards were: 200 μg/mL β-galactosidase (MW 116,000), 200 μg/mL phosphorylase B (MW 94,000), 200 μg/mL BSA (MW 68,000), 900 μg/mL γ-globulin (H-chain MW 50,000, L-chain MW 23,500), 300 μg/mL OVA (MW 43,000) and 300 μg/mL RNase A (MW 13,700). They were dissolved in 10 m*M* Tris-Cl, pH 8.0, 1%SDS, 0.1% β-mercaptoethanol, 20% sucrose and heated in a boiling water bath for 10 min.

When electrophoresis was complete, the gels were usually cut in half. SDS was extracted from one half with 40% methanol, 7% acetic acid overnight at room temperature (M. A. Pickard, personal communication) and stained for protein using 0.1% coomassie blue in 25% isopropanol, 10% acetic acid (Fairbanks *et al*, 1971). SDS was extracted from the other half of the gel using 0.1 *M* Tris-Cl, 1% Triton X-100, pH 7.0 overnight at room temperature in preparation

for an activity stain for Mg^{2+}-independent endonuclease.

Polyacrylamide Gel Activity Stain

The following method, suggested by K. L. Roy (personal communication), was used to determine the position of the Mg^{2+}-independent endonuclease in polyacrylamide gels. After removal of SDS from the polyacrylamide gel, the gel was incubated in 90 m*M* Tris-Cl, 25 m*M* potassium phosphate, 0.2 *M* KCl, 20 m*M* EDTA, pH 7.0 (Mg^{2+}-independent endonuclease reaction buffer) for at least 1 hr at room temperature. It was then laid onto a 0.5% agarose gel (14.5 cm x 14 cm x 1.5 mm) containing 0.2 mg/mL poly(A), covered with saran wrap and incubated at 37°C, usually for 6 hrs. The agarose gel was stained with 0.2% Toluidine Blue O in 0.5% acetic acid for at least 5 min and de-stained by repeated changes of 0.5% acetic acid.

Inhibition of Protein Synthesis by Cycloheximide

S. cerevisiae was grown overnight in yeast extract-glucose broth to an OD_{600} of about 10. The cells were harvested by centrifugation, washed twice with 50 m*M* potassium acetate, pH 5.0 and suspended in this medium to an OD_{600} of 5. To ensure that protein synthesis was inhibited, the following procedure was followed. After addition of 0.1, 0.5 and 1.0 mg/mL cycloheximide, the cells were incubated for 5 min before ^{14}C-leu (308 μCi/μmol) was added to a final concentration of 0.1 μCi/mL. At various times 0.2 mL samples were taken and added to 1.8 mL ice cold 5.5% PCA and

incubated on ice for at least 5 min. The samples were then placed in a boiling water bath for 10 min, cooled quickly and filtered through #3 Whatman filter paper disks. The disks were washed twice with 5 mL cold 5% TCA, air dried and counted in 5 mL Brays scintillation fluid (Bray, 1960).

End Products of the Mg^{2+}-independent Endonuclease

To determine the degradation products of the Mg^{2+}-independent endonuclease, 3 units of purified enzyme isolated from stationary phase cells was added to 20 mL of 50 m*M* Tris-Cl, 1 m*M* EDTA, 1 mg/mL poly(A), pH 7.0. This was incubated at 37°C for 5 hrs and samples were taken to follow the degradation of the substrate. Two hundred μL of the reaction mixture were added to 100 μL of 12% PCA and incubated on ice for 20 min. The precipitate was sedimented by centrifugation in a Beckman microfuge for 1 min. Two hundred μL of the supernatant were diluted to 1 mL in 4% PCA and the absorbance was determined at 260 nm. The reaction was stopped by incubation in a boiling water bath for 5 min. Five mL of these products were applied to a column of DEAE-cellulose as described in the following section. The peak fractions were pooled and treated with snake venom phosphodiesterase I or alkali to determine if the degradation products were 3'- or 5'-phosphate terminated. The phosphodiesterase I assay was carried out after $MgCl_2$ was added to the degradation products to give a final concentration of 8 m*M* and the pH was adjusted to 9.3. Excess

phosphodiesterase I was added and the solution was incubated at 37°C for 30 and 90 min. The reaction was stopped by placing the assay solution on ice. The alkali treatment was carried out in 0.15 *M* KOH at 37 °C for 6 hrs. The reaction was stopped by adjusting the pH to 7.0 with 60% PCA followed by cooling on ice for 15 min. The precipitate which formed was removed by centrifugation at 12,000xg for 10 min.

These reaction products were subjected to descending paper chromatography on Whatman #40 filter paper strips. The solvent systems used were isobutyric acid:*M* NH_4OH (5:3) and 0.1 *M* sodium phosphate (pH 6.8):$(NH_4)_2SO_2$: n-propanol (100 mL:60 g:2 mL). The standards were 2' AMP, 3' AMP, 5' AMP and adenosine. After chromatography, the spots were visualized under short wave ultraviolet light.

DEAE-Cellulose Chromatography of Mg^{2+}-independent Endonuclease Degradation Products

Chromatography of the degradation products was carried out on a 1.2x15 cm column of DEAE-cellulose equilibrated with 20 m*M* NH_4HCO_3 pH 8.3 at 4°C. The degradation products were diluted 5-fold and the pH was adjusted to 8.3 before the solution was applied to the column. The column was eluted with 1 column volume of 20 m*M* NH_4HCO_3 followed by a linear gradient consisting of about 2.5-3 column volumes each of 20 m*M* NH_4HCO_3 and 0.6 *M* NH_4HCO_3, pH 8.3. Fractions of 1 mL were collected at a flow rate of 1 mL/min. The nucleotide content of the fractions was determined by

measuring the absorbance at 260 nm.

Analytical Methods

The method of Bradford (1976) was used for the determination of protein in cell fractions using OVA as the standard. Measurement of the absorbance at 280 nm was used to determine the protein content of column fractions (Warburg and Christian, 1941).

DNA was determined by the method of Stewart (1975). The extraction procedure is summarized below. Samples were treated with an equal volume of 0.5 *M* PCA for 15 min at 0°C and then centrifuged. This was repeated once after the pellet was resuspended to the original volume with cold water. The pellet was then suspended to the original volume with 0.5 *M* PCA. This suspension was incubated at 70°C for 15 min, cooled on ice and centrifuged. The pellets were treated twice more with 0.5 *M* PCA at 70°C for 15 min and all of the supernatants from these treatments were pooled. The DNA content of the supernatant fraction was assayed with the diphenylamine reagent using calf thymus DNA as the standard.

III. RESULTS

A. Growth, Sporulation and Germination of *Saccharomyces cerevisiae* SK-1

Growth

S. cerevisiae was cultured routinely in yeast extract-glucose broth and had a generation time of about 90 min during exponential growth, as seen in Fig 2. When exponential phase cells were required, they were harvested at an OD_{600} of 4 to 6. Stationary phase cells were harvested when the culture had an OD_{600} of greater than 18.

Sporulation

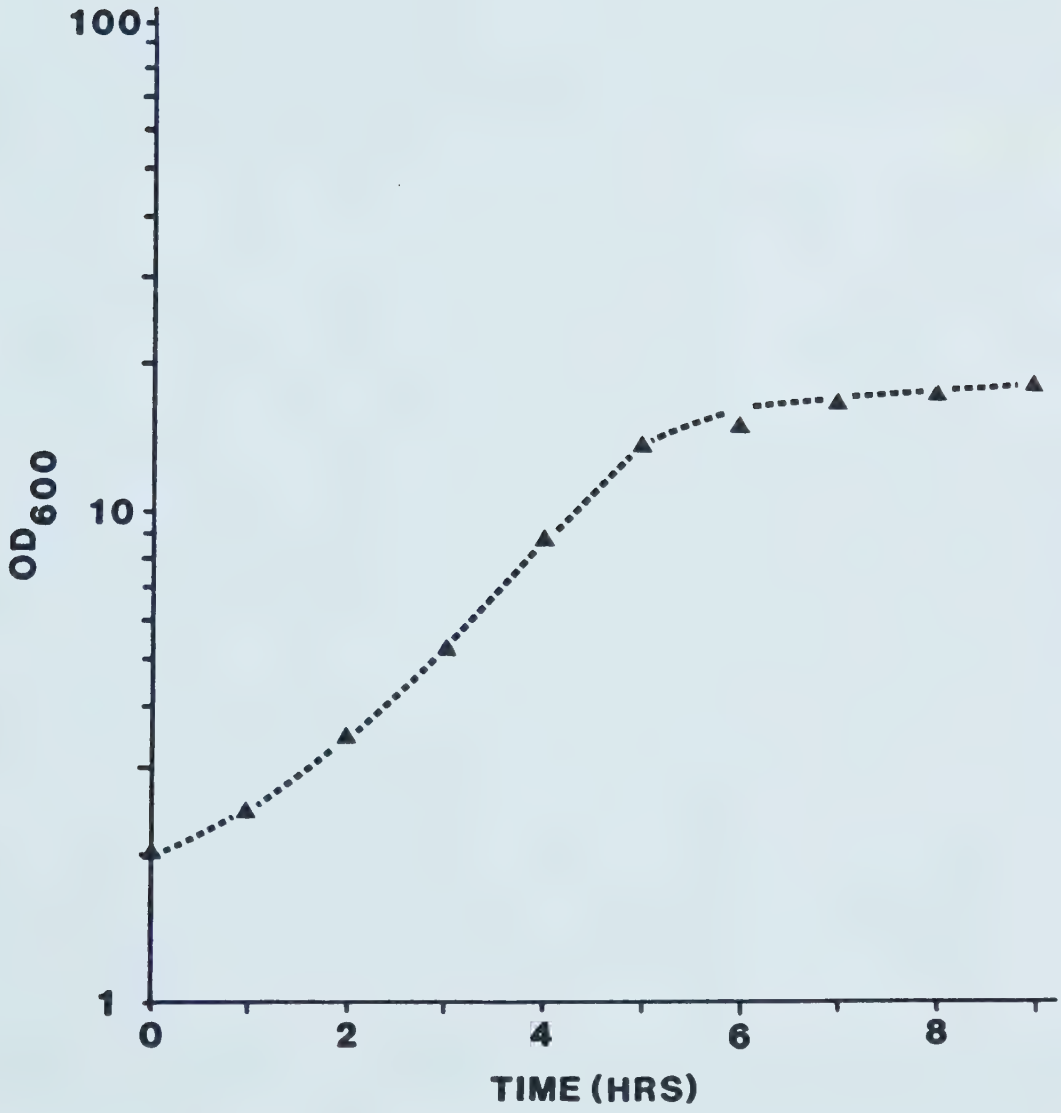
Saccharomyces cerevisiae SK-1 was used in this project because it sporulates efficiently (Kane and Roth, 1974). However, I found that isolates from a culture varied widely with respect to their ability to sporulate. Two of these isolates yielded ascospores to greater than 90% of the total cell population after 24 hrs and one was selected for all further experiments.

It has been noted by some workers that the timing and, perhaps, the extent of ascospore formation are dependent upon the cell density in the sporulation medium (Fowell, 1969). A density of 1 to 2.5×10^7 cells/mL has been used for efficient sporulation (Haber and Halvorson, 1972; Hopper *et al*, 1974; Marmiroli *et al*, 1983). To determine the maximum

FIGURE 2

Growth of *Saccharomyces cerevisiae* SK-1

S. cerevisiae SK-1 was grown in yeast extract-glucose broth at 30°C with shaking at 250 rpm. The medium was inoculated to an optical density of about 2 using a culture grown to stationary phase. Cell growth was measured by optical density at 600 nm after dilution into water.


100
10
1
OD_{600}
0
2
4
6
8
TIME (HRS)

cell density which could be used, *S. cerevisiae* was inoculated into unbuffered sporulation medium to various densities. Sporulation was carried out as described in the methods section and the results are shown in Fig 3. The onset of sporulation was unaffected up to $5x10^7$ cells/mL, but at $10x10^7$ cells/mL it was delayed about 2 hrs, although the extent of ascospore formation was the same in all cases. Therefore, all sporulation experiments were carried out at a density of $5x10^7$ cells/mL.

As yeast sporulate, the pH of the unbuffered sporulation medium rises very quickly from 7 to 9 (Wejksnora and Haber, 1976). While Mills (1972) reported that sporulation is inhibited in buffered sporulation medium, McCusker and Haber (1977) showed that several buffers could be used to stabilize the pH without affecting ascospore formation. To determine if this was true for the yeast used here, sporulation was carried out in unbuffered sporulation medium (pH 7.1), and media buffered with either 0.2 *M* MOPS (pH 6.6) or 0.2 *M* PIPES (pH 6.1). As shown in Fig 4, the pH of the buffered sporulation media showed virtually no change, while the pH of the unbuffered medium quickly increased more than 1.5 units. MOPS was chosen for use in subsequent experiments because the pH was closest to that of the unbuffered sporulation medium and because sporulation was delayed less than when PIPES was used as the buffer.

The progression from a budding vegetative cell, to immature ascospores, to the mature asci as *S. cerevisiae*

FIGURE 3

Sporulation of *S. cerevisiae* SK-1 at Increasing Cell Densities

Cells were harvested from presporulation medium and inoculated into unbuffered sporulation medium at increasing cell densities. They were incubated at 30°C with shaking at 250 rpm. Samples were taken at the indicated times and the percent spores was determined by counting approximately 200 cells under the phase contrast microscope.

1×10^7 cells/mL: •——•

2×10^7 cells/mL: O·- - -O

5×10^7 cells/mL: △——△

10×10^7 cells/mL: □·····□

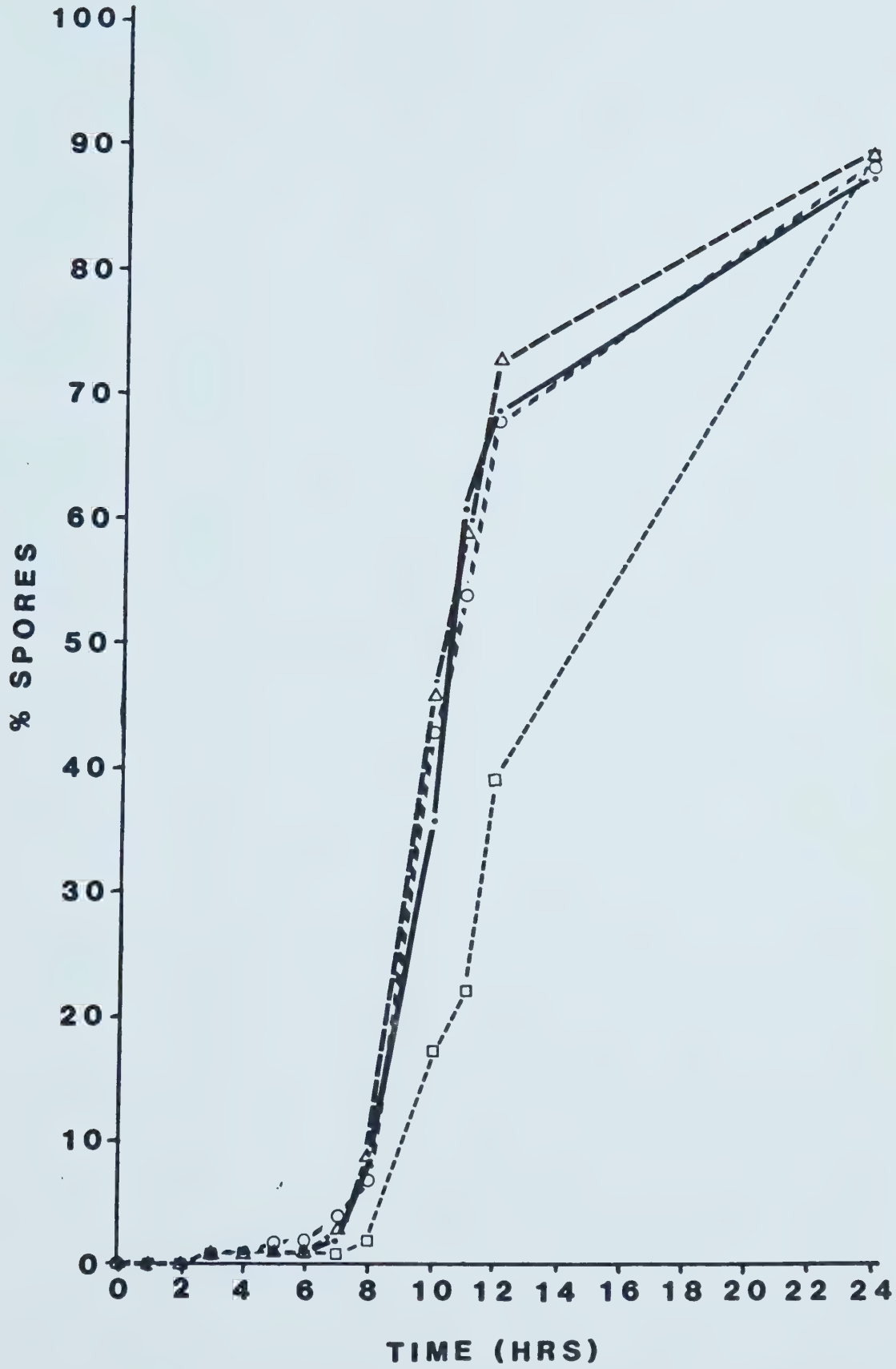

100
90
80
70
60
50
40
30
20
10
0
% SPORES
0
2
4
6
8
10
12
14
16
18
20
22
24
TIME (HRS)

FIGURE 4

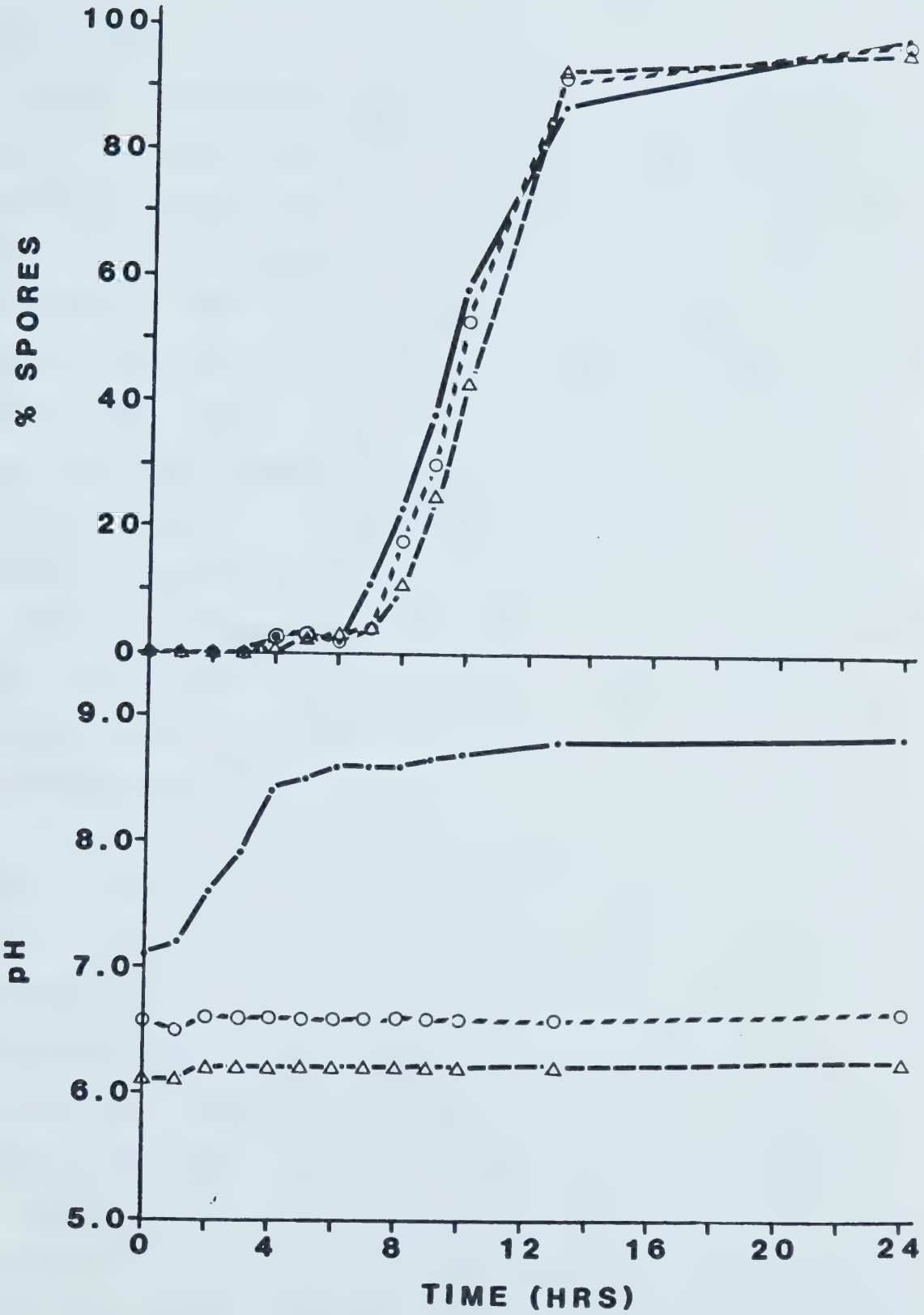
Sporulation of *S. cerevisiae* SK-1 in Buffered and Unbuffered Sporulation Medium

Cells harvested from presporulation medium were inoculated into 1% potassium acetate, pH 7.1 and 1% potassium acetate buffered with 0.2 *M* MOPS, pH 6.6 or 0.2 *M* PIPES, pH 6.1 to a final cell density of 5×10^7 cells/mL. The cells were incubated at 30°C with shaking at 250 rpm. Samples were taken at the indicated times and the percent spores and pH of each were determined.

Unbuffered Sporulation Medium: •——•

Medium Buffered with MOPS: O- - -O

Medium Buffered with PIPES: Δ•——Δ


100
80
60
40
20
0
% SPORES
9.0
8.0
7.0
6.0
5.0
pH
0
4
8
12
16
20
24
TIME (HRS)

sporulates is shown in Plate 1.

Germination

Yeast ascospores proceed from a dormant to a vegetative, metabolically active state as they germinate. To determine the nuclease activities in these cells, they were treated with Zymolyase 60,000 to remove the ascus coat as described in the methods section. This was done for three reasons: the spore suspension could be broken quickly after samples were taken, germination could be followed more readily by phase contrast microscopy and cells which had not sporulated could be removed by this procedure. A typical germination experiment is presented in Fig 5 which shows the increase in vegetative cells after inoculation of free spores into yeast extract-glucose broth. Germination proceeded very quickly and synchronously and was more than 90% complete after 4 hrs.

Initially germination was determined by counting the number of cells with buds, but this was abandoned for two reasons. The first was that not all of the cells will have buds, resulting in an underestimation of the percentage of vegetative cells. The second was that metabolic activity would probably increase to vegetative levels well before bud emergence. Therefore, to determine the point of germination, the appearance of the cells under the phase contrast microscope was used. The spores are very refractile and appear light, while the vegetative cells are non-refractile

PLATE 1

Phase Contrast Photomicrographs of *S. cerevisiae* SK-1 at Different Stages of Sporulation

Cells were collected, washed and resuspended into sporulation medium to a final cell density of 5×10^7 cells/mL. Wet mounts of the cell suspension were photographed at various times during sporulation.

A. Vegetative cell at zero time in sporulation showing a bud. Magnification=730

B. Immature spores contained in an ascus. Magnification=560

C. Mature ascospores (⟷) with an unsporulated cell (▷) Magnification=800

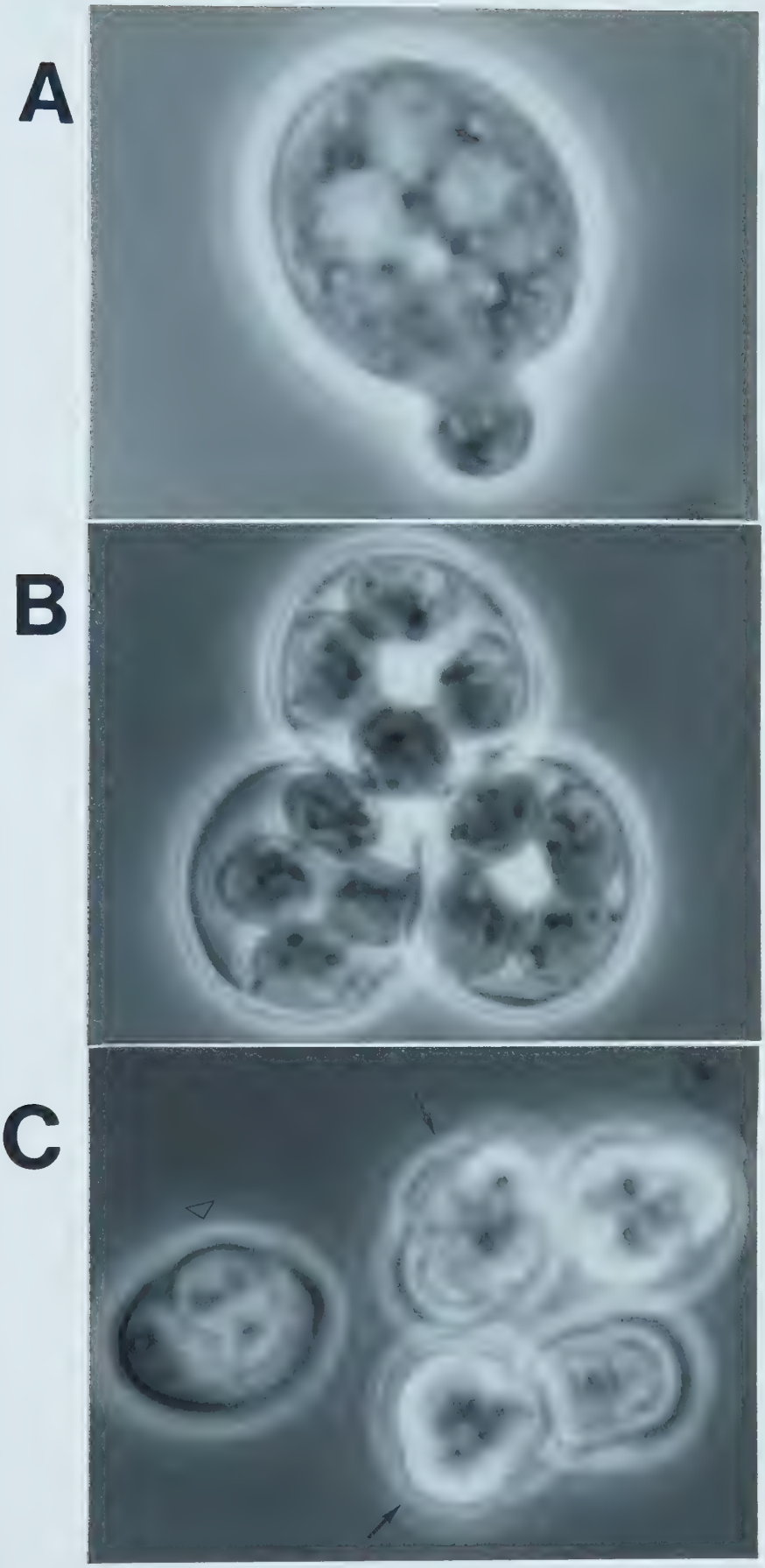
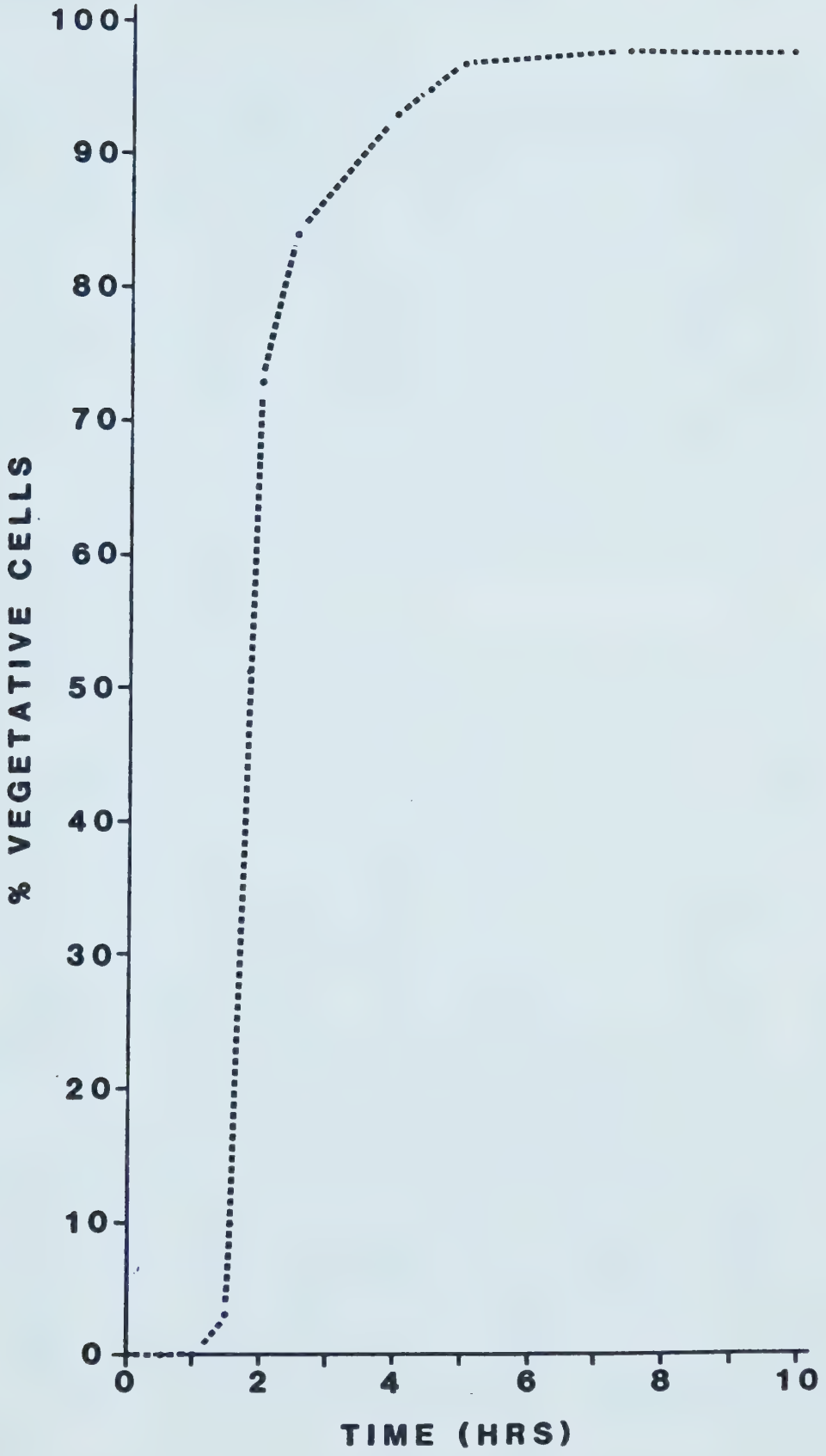

A
B
C

FIGURE 5

Germination of S. *cerevisiae* SK-1 Spores

Spores were inoculated into yeast extract-glucose broth to a density of 5×10^7 cells/mL after being released from the ascus. They were incubated at 30°C with shaking at 250 rpm. Outgrowth was followed under the phase contrast microscope by counting the number of light cells (spores) and dark cells (vegetative cells)


100
90
80
70
60
50
40
30
20
10
0
% VEGETATIVE CELLS
0
2
4
6
8
10
TIME (HRS)

and appear dark, as shown in Plate 2. This allowed more objective observation of spore germination.

B. Differential Assays of the Nucleic Acid-Degrading Enzymes

Three nucleic acid-degrading enzymes were investigated in this project. These enzymes are called Mg^{2+}-dependent endonuclease (von Tigerstrom, 1982), Mg^{2+}-independent endonuclease (Ohtaka *et al*, 1963; Nakao *et al*, 1968) and a Mg^{2+}-dependent 5'-exonuclease (Stevens, 1980). It was hoped that these three enzymes could be assayed fairly specifically in crude cell extracts. However, the extent to which each nuclease might contribute to the activity of the others had to be determined.

The substrate used in the assay of all three enzymes was poly(A). The Mg^{2+}-dependent 5'-exonuclease required the use of ^{3}H-poly(A) due to the low activity of this enzyme. Although the enzymes could not be differentially assayed according to their substrate, they could be distinguished according to the characteristics listed in Table 3. The divalent metal requirement was important since the Mg^{2+}-independent endonuclease was completely active in the presence of high concentrations of EDTA, whereas the other two enzymes were completely inactive in the absence of Mg^{2+}.

The pH of the assay solution was used in part to differentiate the Mg^{2+}-dependent 5'-exonuclease activity from the other nucleases, since its optimum pH is 8.0-8.5 (Stevens, 1980). At this pH the Mg^{2+}-independent and

PLATE 2

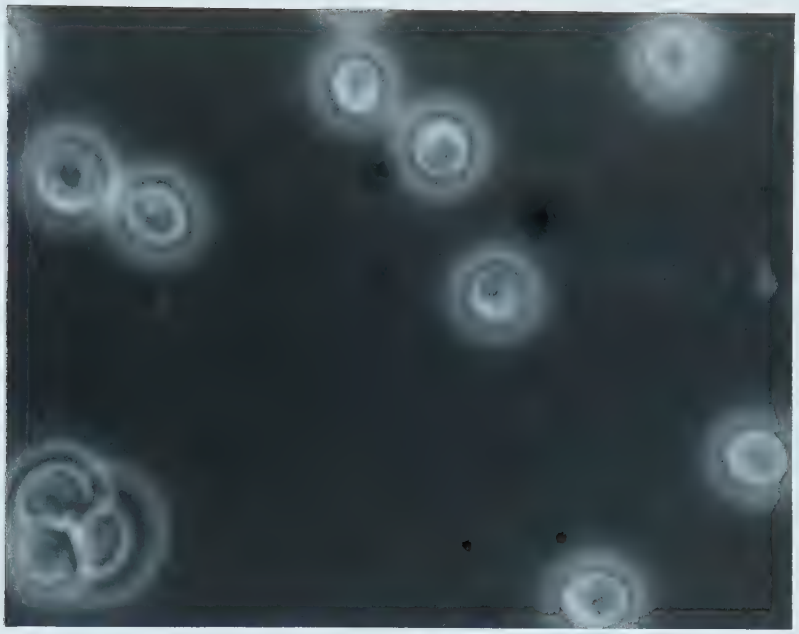
Phase Contrast Photomicrographs of Germinating Spores of *S. cerevisiae* SK-1

The spores were released from the ascus and inoculated into yeast extract-glucose broth to a final cell density of 5×10^7 cells/mL as described in the methods section. Samples of the spore suspension were photographed after preparation of wet mounts.

A. Individual spores at zero time of germination. The spores appear very light under the phase contrast microscope. Magnification=520

B. Shows a mixture of ungerminated spores (▷) and vegetative cells (→). The vegetative cells appear dark under the phase contrast microscope. Magnification=640

A



B

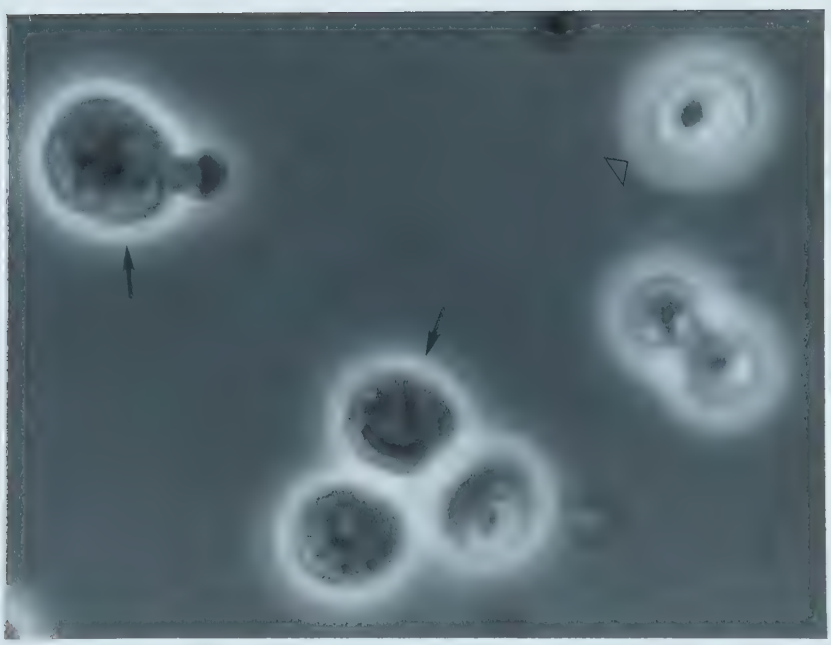


TABLE 3

Summary of the Characteristics used to Differentiate the Mg^{2+}-independent Endonuclease, Mg^{2+}-dependent Endonuclease and Mg^{2+}-dependent 5'-exonuclease

	Mg^{2+}-independent Endonuclease	Mg^{2+}-dependent Endonuclease	Mg^{2+}-dependent 5'-exonuclease
Mg^{2+} Required	No	Yes	Yes
Assay pH	7.0	7.0	8.0
Effect of Zwittergent 3-14	None	Activates	Inhibits

Mg^{2+}-dependent endonucleases have very low activity, as seen in Table 4. However, the Mg^{2+}-dependent 5'-exonuclease assay using ^{3}H-poly(A) is very sensitive and even low activities of the other enzymes could still interfere. The effect of Zwittergent 3-14 on each of the enzymes overcame this difficulty. While this detergent is required to solubilize and activate the Mg^{2+}-dependent endonuclease (von Tigerstrom, 1982), it had no effect on the Mg^{2+}-independent endonuclease and completely inhibited the Mg^{2+}-dependent 5'-exonuclease. To confirm this, the Mg^{2+}-independent endonuclease and Mg^{2+}-dependent 5'-exonuclease activities were separated by heparin agarose chromatography as described in the methods section. The elution of the enzymes is shown below (Fig 7). Assays of the fractions using the reaction mixture for the Mg^{2+}-dependent 5'-exonuclease containing ^{3}H-poly(A) but not Zwittergent 3-14 revealed two peaks of activity. Only the first peak was seen in the presence of the detergent so this activity was likely due to the Mg^{2+}-independent endonuclease. To confirm this, the mode of attack of the three enzymes was verified and is shown in Table 5. When the mode of attack of the two peaks of activity was determined, it was found that peak I was an endonuclease while peak II, which was not seen in the presence of detergent, was an exonuclease. Therefore the Mg^{2+}-independent endonuclease (peak I) cross-reacts in the assay of the Mg^{2+}-dependent 5'-exonuclease (peak II) but the two enzymes can be distinguished by adding detergent.

TABLE 4

Activity of the Mg^{2+}-independent Endonuclease and Mg^{2+}-dependent Endonuclease at pH 8.0[a]

	pH	Activity (U/mL)	%Activity
Mg^{2+}-independent Endonuclease	7.0	0.654	100
	8.0	0.012	1.8
Mg^{2+}-dependent Endonuclease	7.0	1.683	100
	8.0	0.012	0.7

[a]The enzymes used were purified by extraction of cell extracts and heparin agarose chromatography

TABLE 5

Mode of Hydrolysis of the Mg^{2+}-independent Endonuclease, Mg^{2+}-dependent Endonuclease and Mg^{2+}-dependent 5'-exonuclease[a]

	Nuclease Activity (U/mL)			Mode of Hydrolysis
	12% PCA	12% PCA Uranyl Acetate	Relative Activity	
Mg^{2+}-independent Endonuclease	0.602	0.237	1:0.394	Endonucleolytic
Mg^{2+}-dependent Endonuclease	1.458	0.612	1:0.420	Endonucleolytic
Mg^{2+}-dependent 5'-exonuclease ($x10^4$)	6.036	5.974	1:0.990	Exonucleolytic

[a]The enzymes used were purified by extraction of cell extracts and heparin agarose chromatography

In a similar experiment, the Mg^{2+}-dependent endonuclease was purified by heparin agarose chromatography. It was found that this enzyme was not active under the conditions of the Mg^{2+}-dependent 5'-exonuclease assay.

C. Separation of the Nucleic Acid-Degrading Enzymes

Preparation of Cell Extracts

Before changes in nuclease activities could be determined, it was necessary to develop methods to break the cells and to extract the enzymes quickly and efficiently. Two methods were considered. The first method involved the preparation of sphaeroplasts which were disrupted by osmotic shock followed by brief sonication. The second method involved breaking the cells directly with the French pressure cell.

Preparation of Sphaeroplasts

Sphaeroplasts were prepared as described in detail in the methods section and the formation of sphaeroplasts was considered complete when the OD_{600} of the diluted suspension decreased about 90-95%. As shown in Fig 6, it required incubation at 30°C for at least 25 and 50 min to produce complete sphaeroplasts of exponential phase and stationary phase cells, respectively. During this time, the metabolism of the cells would have undoubtedly changed, which is a serious drawback of this method.

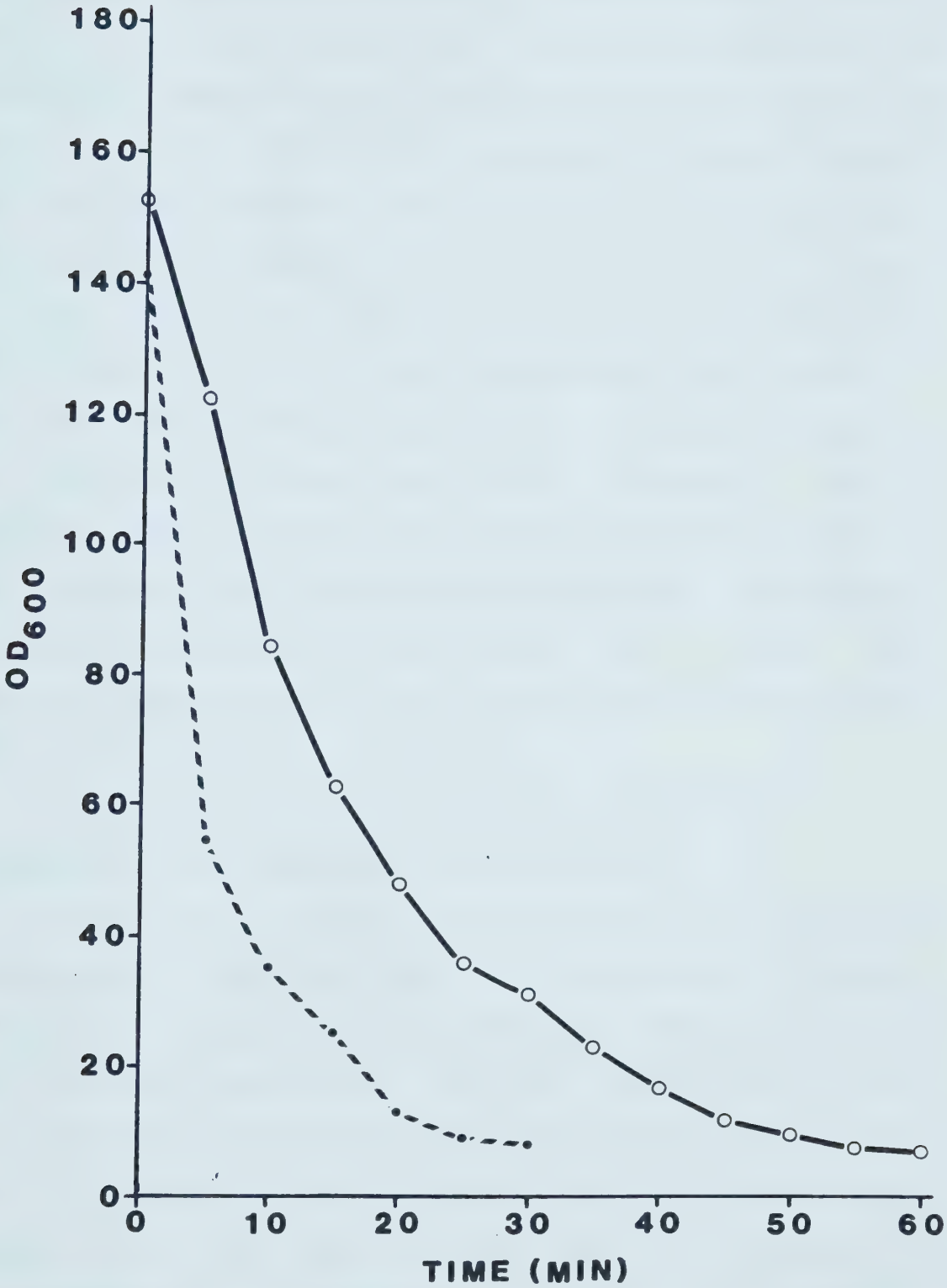
FIGURE 6

Sphaeroplast Formation of *S. cerevisiae* SK-1 Exponential- and Stationary Phase Cells

Exponential- and stationary phase cells were used to prepare sphaeroplasts as described in the methods section. Sphaeroplast formation was followed by diluting the cell suspension into water and determining the decrease in optical density at 600 nm.

Exponential Phase Cells: •–––•

Stationary Phase Cells: O——O


180
160
140
120
100
80
60
40
20
0
OD_{600}
0
10
20
30
40
50
60
TIME (MIN)

Preparation of French Pressure Cell Lysate

To determine the efficiency of breakage of the yeast by this method, cells were counted in a haemacytometer before and after passage through the French pressure cell. The breakage obtained with sporulating and germinating cells is shown in Table 6. Time (hrs) represents the number of hours the cells were incubated under sporulation or germination conditions. The proportion of broken cells was, in almost all cases, greater than 90%. The two exceptions were the diploid strain at 10 hrs under sporulation conditions and at zero time under germination conditions. Therefore, it was not possible to break the spores efficiently and to determine their nuclease activities accurately. In spite of these difficulties, this method of preparing cell extracts was used throughout this project since it is faster than preparing sphaeroplasts and did not require incubation at 30°C.

Extraction of Nucleases from Cell Extracts

To determine the characteristics of the nucleases being investigated, it was desirable to develop methods to extract and separate the enzymes. To optimize the separation of the major nuclease activities, the cell extracts were treated with different concentrations of KCl. There was little effect on the solubility of the Mg^{2+}-dependent endonuclease between 0.5 *M* and 2.0 *M* KCl but the amount of Mg^{2+}-independent endonuclease and Mg^{2+}-dependent

TABLE 6

Efficiency of Breakage of *S. cerevisiae* SK-1 Sporulating and Germinating Cells by the French Pressure Cell

Time(Hrs)	% Unbroken Cells			
	Sporulating Cells		Germinating Cells	
	Haploid	Diploid	Haploid	Diploid
0	4.5	1.1	7.8	76.2
2.5	1.7	0.8	8.5	8.5
5	1.7	1.7	5.7	2.2
7.5	1.9	7.1	5.0	2.6
10	2.9	41	4.6	2.6

5'-exonuclease extracted increased considerably. Therefore, in the preparation of cell extracts, KCl was added to 2 *M* to solubilize the nucleases, and the Mg^{2+}-dependent endonuclease was separated from the other two enzymes by centrifugation at 100,000xg as described in the methods section. More than 90% of the Mg^{2+}-dependent endonuclease was found in the pellet fraction while the Mg^{2+}-independent endonuclease and the Mg^{2+}-dependent 5'-exonuclease activities were found almost exclusively in the supernatant. If the cell extracts were not treated with KCl, all three nucleases were found in the pellet after centrifugation.

Heparin Agarose Chromatography

The pellet and supernatant fractions of the cell extracts were analysed by chromatography on heparin agarose as described in the methods section. A typical column profile of the 100,000xg supernatant is shown in Fig 7 and chromatography of the 100,000xg pellet solubilized with 0.2% Zwittergent 3-14 is shown in Fig 8.

Two peaks of activity, designated peak I and II, were observed in Fig 7 when the assay for the Mg^{2+}-dependent 5'-exonuclease activity was carried out in the absence of Zwittergent 3-14. Peak I was insensitive to the detergent and eluted precisely with the Mg^{2+}-independent endonuclease. Peak II was inhibited by Zwittergent 3-14 and this peak was due to the activity of the Mg^{2+}-dependent 5'-exonuclease. The profile of the Mg^{2+}-dependent 5'-exonuclease activity

FIGURE 7

Heparin Agarose Chromatography of *S. cerevisiae* SK-1 Exponential Phase Cell Extract 100,000xg Supernatant

The cell extract 100,000xg supernatant was dialysed overnight at 0-4°C against two changes of buffer 2 without Zwittergent 3-14. A 4 mL sample was applied to a 1.2x40 cm column of heparin agarose and eluted as described in the methods section. Fractions of 1.8 mL each were collected at a flow rate of 0.8-0.9 mL/min and assayed for nuclease activity and protein as described in the methods section.

Mg^{2+}-independent Endonuclease (x10^2): •----•

Mg^{2+}-dependent Endonuclease (x10^2): △——△

Mg^{2+}-dependent 5'-exonuclease (x10^4): ■——■

Protein Concentration: ▼- - -▼

KCl Concentration: ----

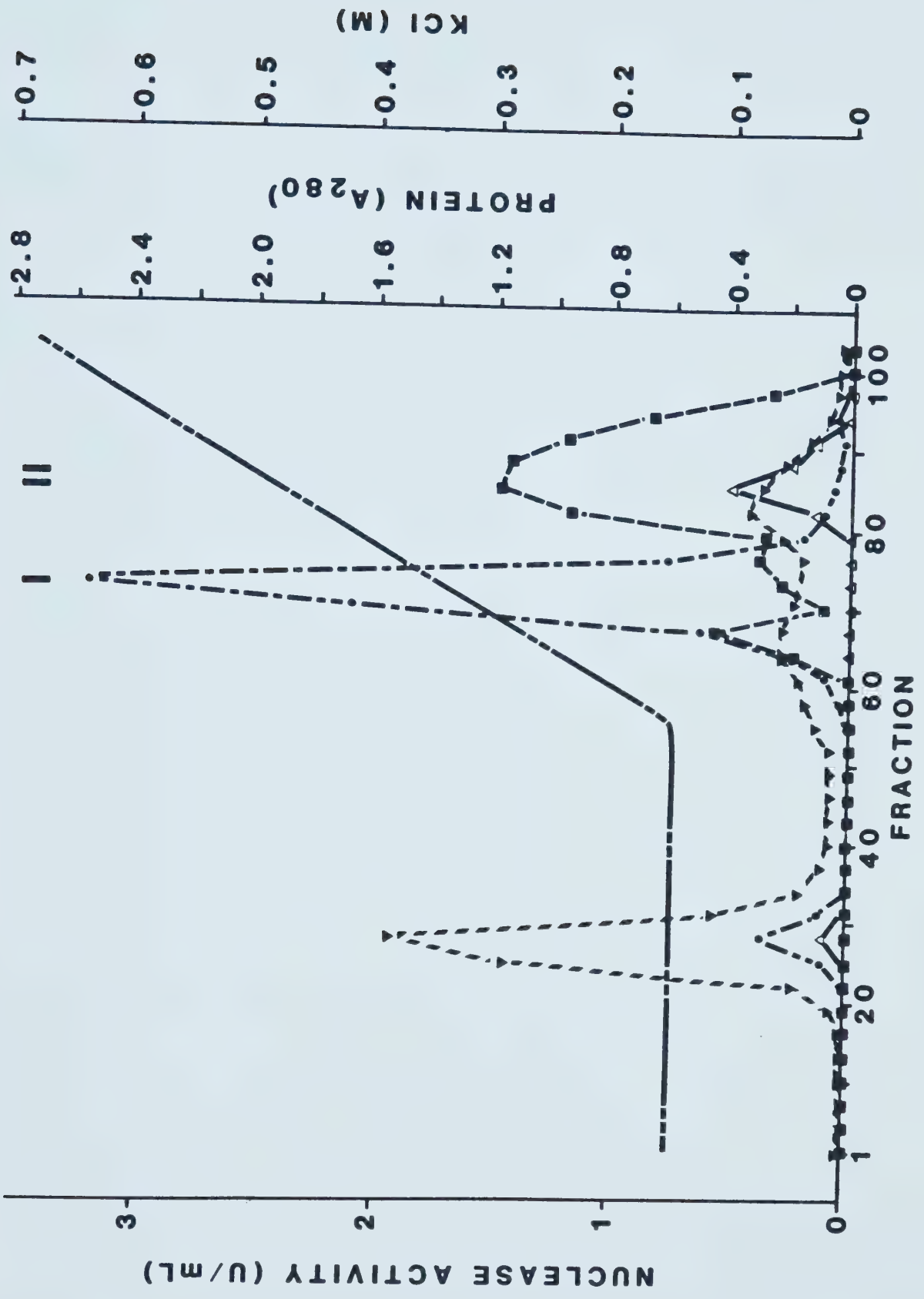

KCl (M)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
PROTEIN (A_{280})
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0
I
II
NUCLEASE ACTIVITY (U/mL)
3
2
1
0
1
20
40
60
80
100
FRACTION

FIGURE 8

Heparin Agarose Chromatography of *S. cerevisiae* SK-1 Exponential Phase Solubilized Cell Extract 100,000xg Pellet

The cell extract 100,000xg pellet was treated with 0.2% Zwittergent 3-14 and dialysed overnight at 8-10°C against two changes of buffer 2. A 4 mL sample was applied to a 1.2x40 cm column of heparin agarose and eluted as described in the methods section. Fractions of 1.8 mL were collected at a flow rate of 0.8-0.9 mL/min and assayed for nuclease activity and protein as described in the methods section. Nuclease activity with ssDNA as substrate was also determined using Total nuclease assay conditions.

Mg^{2+}-independent Endonuclease ($x10^2$): •—-—•

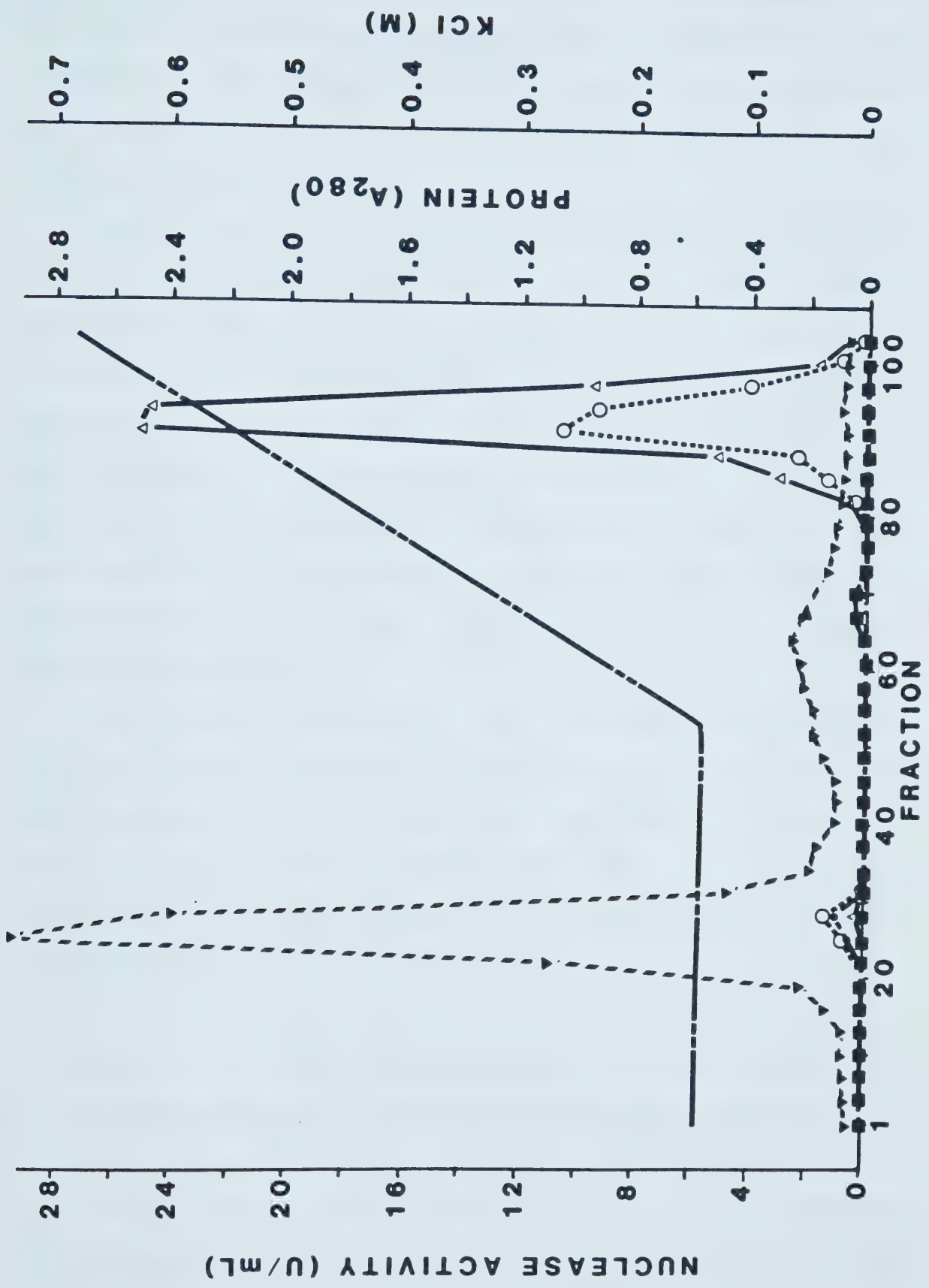
Mg^{2+}-dependent Endonuclease ($x10^2$): △——△

Mg^{2+}-dependent 5'-exonuclease ($x10^4$): ■·—·—·■

ssDNA ($x10^2$): O·····O

Protein Concentration: ▼- - -▼

KCl Concentration: ----


NUCLEASE ACTIVITY (U/mL)
0
4
8
12
16
20
24
28
PROTEIN (A_{280})
0
0.4
0.8
1.2
1.6
2.0
2.4
2.8
KCl (M)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
FRACTION
1
20
40
60
80
100

presented in Fig 7 has been corrected by subtraction as described in the methods section. The fractions were also assayed for Mg^{2+}-independent endonuclease activity using ds- and ssDNA as substrate. Little or no activity was observed in any of the fractions.

Both Figs 7 and 8 show that chomatography on heparin agarose can be used to separate and partially purify these nucleases. They also show that centrifugation of the cell extracts at 100,000xg resulted in a virtually complete separation of the Mg^{2+}-independent endonuclease and Mg^{2+}-dependent 5'-exonuclease activities from the particulate Mg^{2+}-dependent endonuclease. Assay of the fractions with ds- and ssDNA as substrate showed that the Mg^{2+}-dependent endonuclease was fairly active with the ssDNA but not with dsDNA.

From these results it can be seen that the three nuclease activities could be quantitatively separated by centrifugation at 100,000xg and partially purified on heparin agarose. This separation was essential for establishing specific assay conditions for the enzymes as described above.

D. Changes in Activity of the Nucleic Acid-Degrading Enzymes Throughout the Life Cycle of *Saccharomyces cerevisiae*

To determine the possible functions of the Mg^{2+}-independent endonuclease, the Mg^{2+}-dependent endonuclease and the Mg^{2+}-dependent 5'-exonuclease it was

decided to follow the changes in their activities during the cell cycle. The stages which were investigated were the transition from exponential to stationary phase (vegetative growth), sporulation and germination. The following experiments were carried out at least twice and were found to be reproducible.

Vegetative Growth

As the cells proceed from exponential to stationary phase, a variety of metabolic changes occur. As the growth rate slows, the rate of breakdown of macromolecules such as protein and RNA increases (Hopper *et al*, 1974). The nuclease activities at these two growth stages were measured and are shown in Table 7. The increase in activity of the Mg^{2+}-independent endonuclease and Mg^{2+}-dependent 5'-exonuclease from exponential to stationary phase indicates that these two enzymes may be involved in the degradation of RNA. Of the two, the Mg^{2+}-independent endonuclease showed the greatest change suggesting that this enzyme may have a more active role in RNA metabolism. The activity of the Mg^{2+}-dependent endonuclease decreased about 50% as the cells proceeded from exponential to stationary phase indicating that it has a function other than the degradation of RNA, but its role cannot be determined at this point.

TABLE 7

Changes in Nuclease Activities of *S. cerevisiae* SK-1 as the Cells Proceed from Exponential Phase to Stationary Phase

	Nuclease Activity (U/mg)		
	Exponential Phase	Stationary Phase	Relative Activity
Mg^{2+}-independent Endonuclease ($x10^2$)	3.57	13.47	1:3.8
Mg^{2+}-dependent Endonuclease ($x10^2$)	11.66	5.52	1:0.5
Mg^{2+}-dependent 5'-exonuclease ($x10^6$)	1.93	3.82	1:2.0

Sporulation

S. cerevisiae strains which are heterozygous at the mating type locus form asci when placed in a medium containing a non-fermentable carbon source, such as acetate, in the absence of a nitrogen source (Esposito and Klapholz, 1981). To determine if changes in nuclease activities were sporulation specific, a non-sporulating control was required. For this purpose it was decided to use a haploid strain of *S. cerevisiae* SK-1. Its isolation is described in the methods section. Unless stated otherwise the haploid strain was treated in exactly the same manner as the diploid strain.

The changes which occur with respect to the nuclease activities from the diploid and haploid strains under sporulation conditions are shown in Figs. 9 and 10, respectively. In both cell populations, the Mg^{2+}-independent endonuclease and Mg^{2+}-dependent 5'-exonuclease activities increased considerably over the course of the experiment, suggesting that these changes are not sporulation specific, but reflect the starvation conditions which prevailed. Under these conditions, RNA is being continually synthesized and degraded at increasing rates. The increase in the Mg^{2+}-independent endonuclease and Mg^{2+}-dependent 5'-exonuclease activities indicates that these enzymes may function in the degradation of RNA. The Mg^{2+}-dependent 5'-exonuclease showed a larger increase in the diploid strain than the haploid cells, but it is difficult to

FIGURE 9

Changes in Nuclease Activities of Diploid *S. cerevisiae* SK-1 Under Sporulation Conditions

Diploid cells were placed under sporulation conditions at a density of 5×10^7 cells/mL and cell extracts were obtained at 2.5 hr intervals by passing the cells through a French pressure cell at 103 MPa. Nuclease activities were isolated and assayed as described in the methods section. Ascospore formation was followed by phase contrast microscopy as described previously.

Mg^{2+}-independent Endonuclease ($\times 10^2$): •-—-•

Mg^{2+}-dependent Endonuclease ($\times 10^2$): △——△

Mg^{2+}-dependent 5'-exonuclease ($\times 10^4$): ■——■

% Spores: □·····□

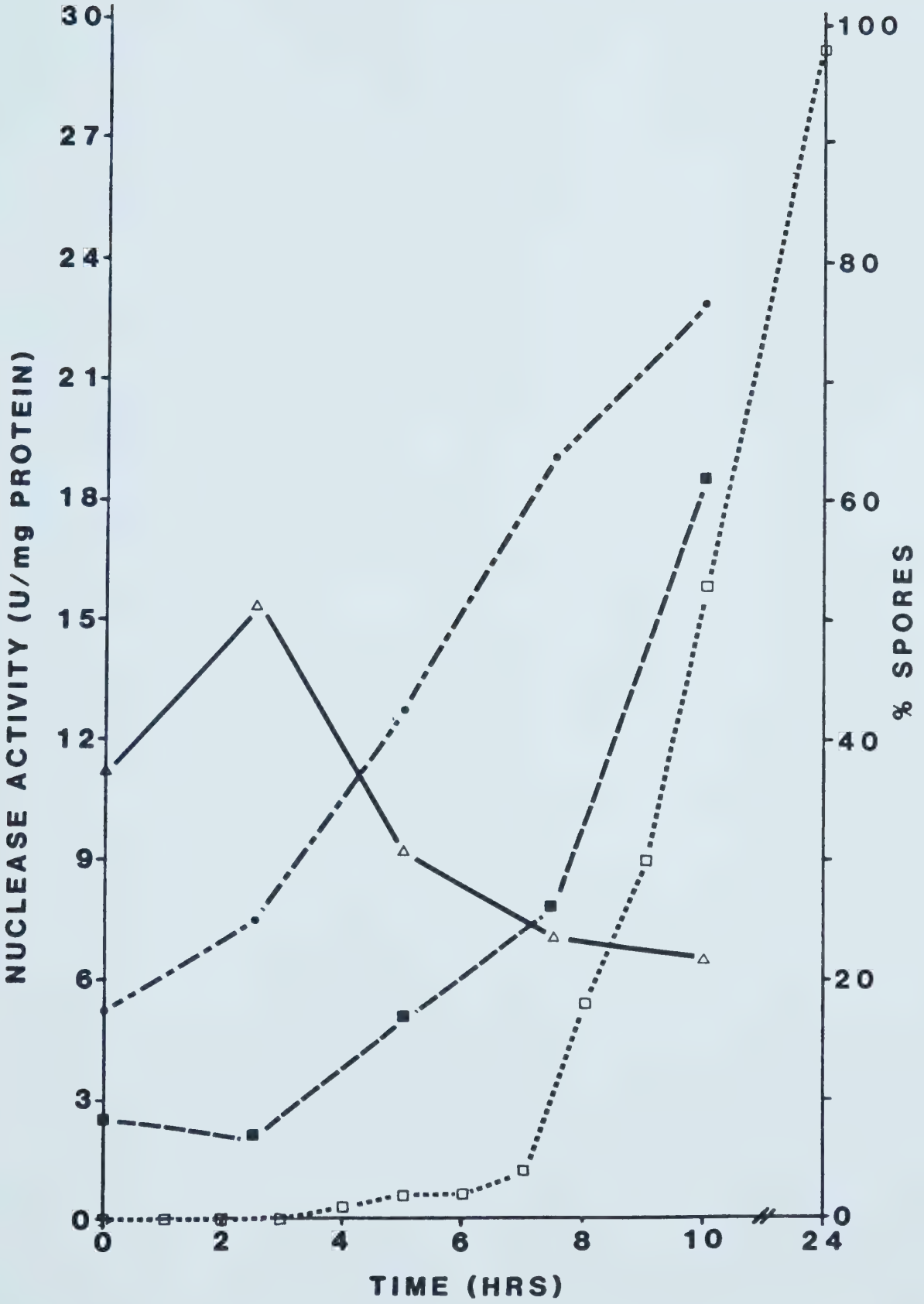

NUCLEASE ACTIVITY (U/mg PROTEIN)
% SPORES
TIME (HRS)
30
27
24
21
18
15
12
9
6
3
0
100
80
60
40
20
0
0
2
4
6
8
10
24

FIGURE 10

Changes in Nuclease Activities of Haploid *S. cerevisiae* SK-1 Under Sporulation Conditions

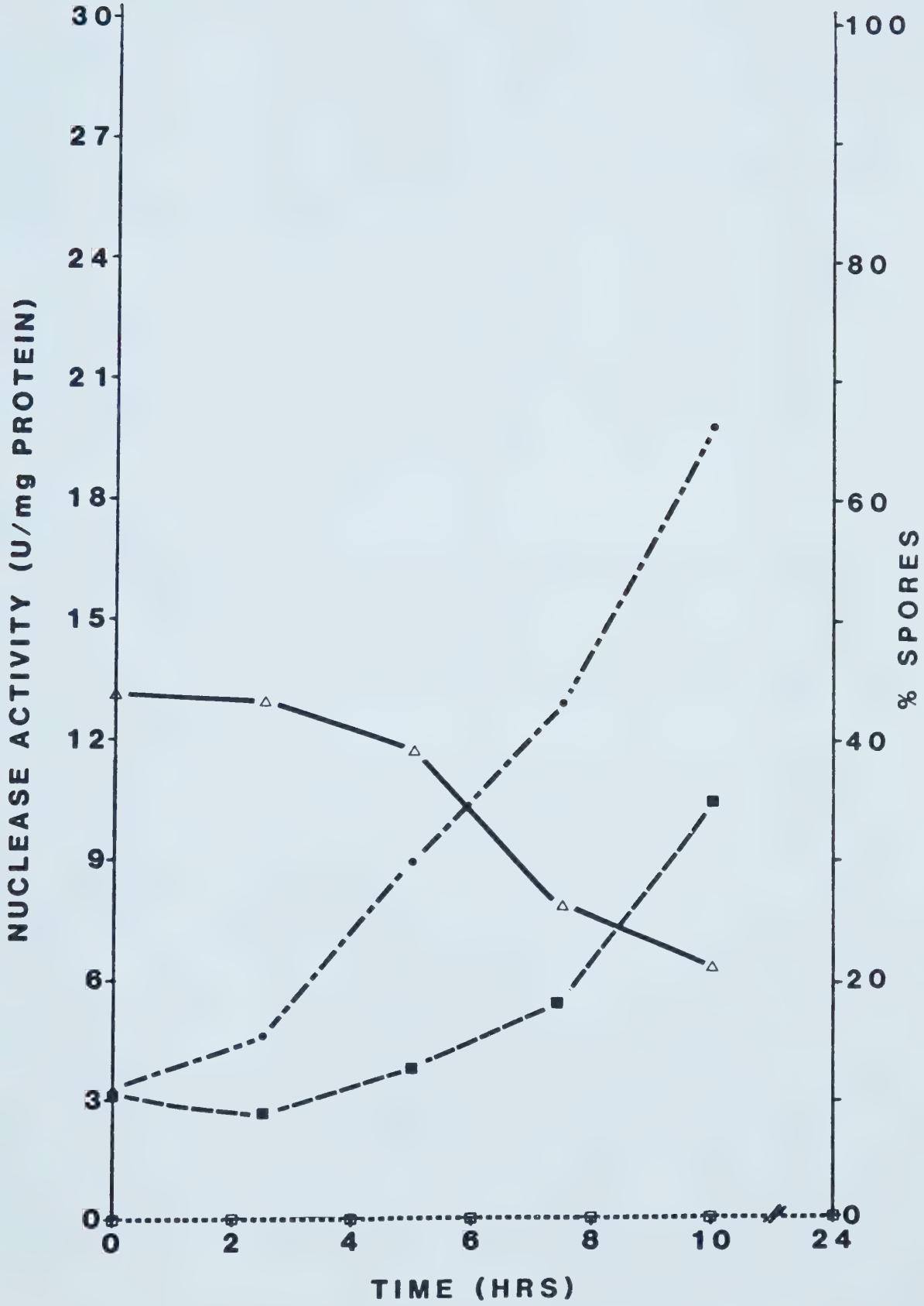
Haploid cells were inoculated into sporulation medium to a density of 5×10^7 cells/mL and cell extracts were obtained at 2.5 hr intervals by passing the cells through a French pressure cell at 103 MPa. The nucleases were isolated and assayed as described in the methods section.

Mg^{2+}-independent Endonuclease ($\times 10^2$): •—•—•

Mg^{2+}-dependent Endonuclease ($\times 10^2$): △——△

Mg^{2+}-dependent 5'-exonuclease ($\times 10^4$): ■—■—■

% Spores: □·····□


NUCLEASE ACTIVITY (U/mg PROTEIN)
% SPORES
TIME (HRS)
30
27
24
21
18
15
12
9
6
3
0
100
80
60
40
20
0
0
2
4
6
8
10
24

determine if this is a sporulation specific event since the same trend was seen in both populations.

The activity of the Mg^{2+}-dependent endonuclease decreased about 50% over the course of the experiment in both cell populations which may reflect a decrease in the number or activity of the mitochondria, degradation of the enzyme, or both. However, in some experiments its activity increased as much as 2-fold in the first few hours of sporulation in the diploid strain. This increase was not observed in the haploid strain which indicates that the Mg^{2+}-dependent endonuclease may have a role in meiosis.

Germination

The germination experiment was carried out as described in the methods section except that the haploid cells were not treated with Zymolyase 60,000 or passed through the French pressure cell.

Changes in the enzyme activities during germination of the spores can be seen in Fig 11. The activity of the Mg^{2+}-dependent endonuclease increased from almost undetectable levels to become the major nuclease activity in the cell while the Mg^{2+}-independent endonuclease and Mg^{2+}-dependent 5'-exonuclease showed little change.

Changes in nuclease activities of the haploid strain after the cells were transfered from sporulation medium to growth medium are shown in Fig 12. The changes in the Mg^{2+}-dependent endonuclease observed here are very similar

FIGURE 11

Changes in Nuclease Activities of *S. cerevisiae* SK-1 Spores Under Germination Conditions

Asci were collected and the spores liberated as described in the methods section. They were inoculated to 5×10^7 cells/mL into yeast extract-glucose broth and cell extracts were obtained at 2.5 hr intervals by passing the cells through a French pressure cell at 103 MPa. Outgrowth was followed by phase contrast microscopy and the nuclease activities were isolated and assayed as described in the methods section.

Mg^{2+}-independent Endonuclease ($\times 10^2$): •---•

Mg^{2+}-dependent Endonuclease ($\times 10^2$): △——△

Mg^{2+}-dependent 5'-exonuclease ($\times 10^4$): ■—·—■

% Vegetative Cells: □·····□

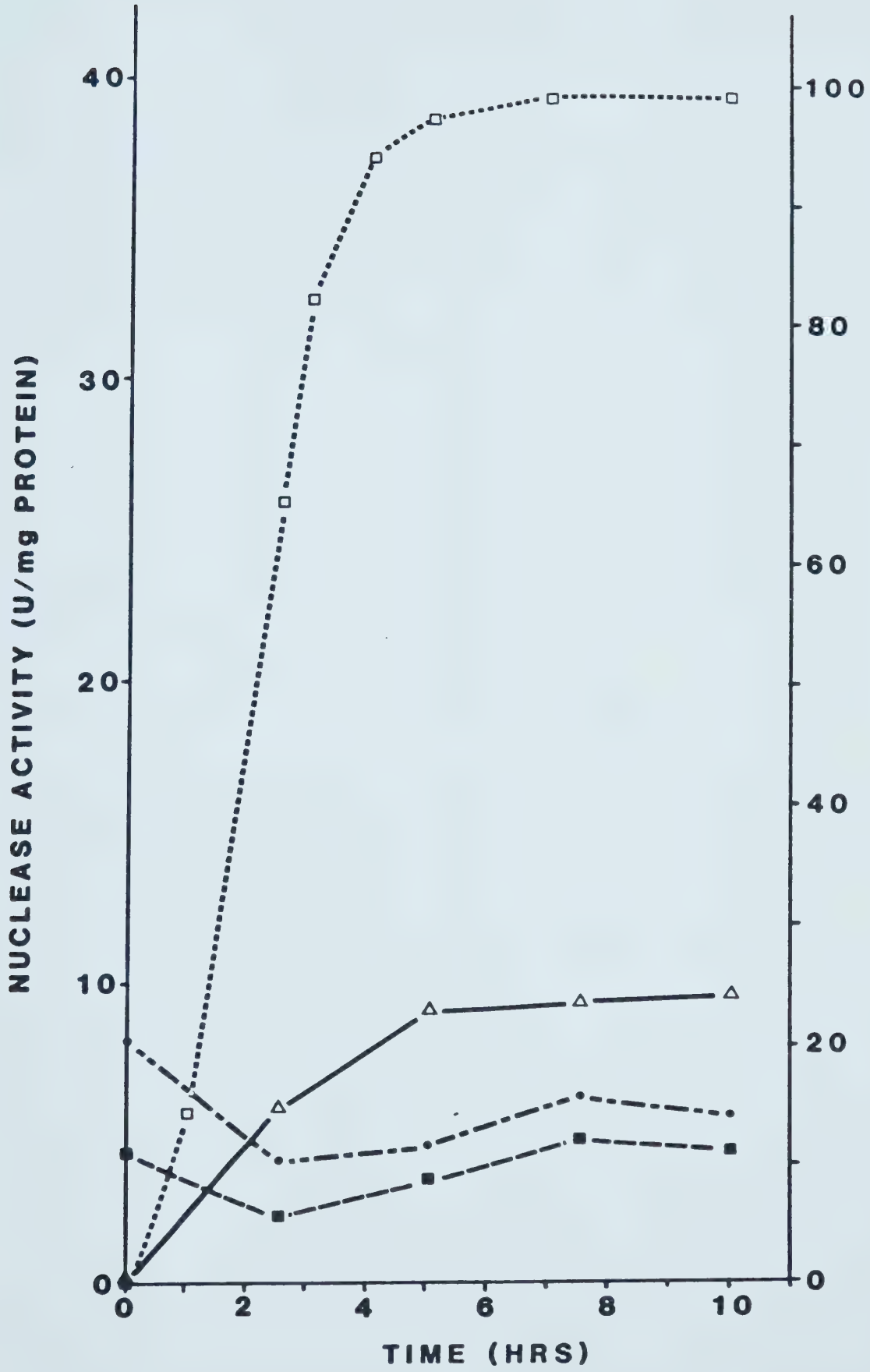

NUCLEASE ACTIVITY (U/mg PROTEIN)
% VEGETATIVE CELLS
TIME (HRS)
0
10
20
30
40
0
20
40
60
80
100
0
2
4
6
8
10

FIGURE 12

Changes in Nuclease Activities of Haploid *S. cerevisiae* SK-1 Under Germination Conditions

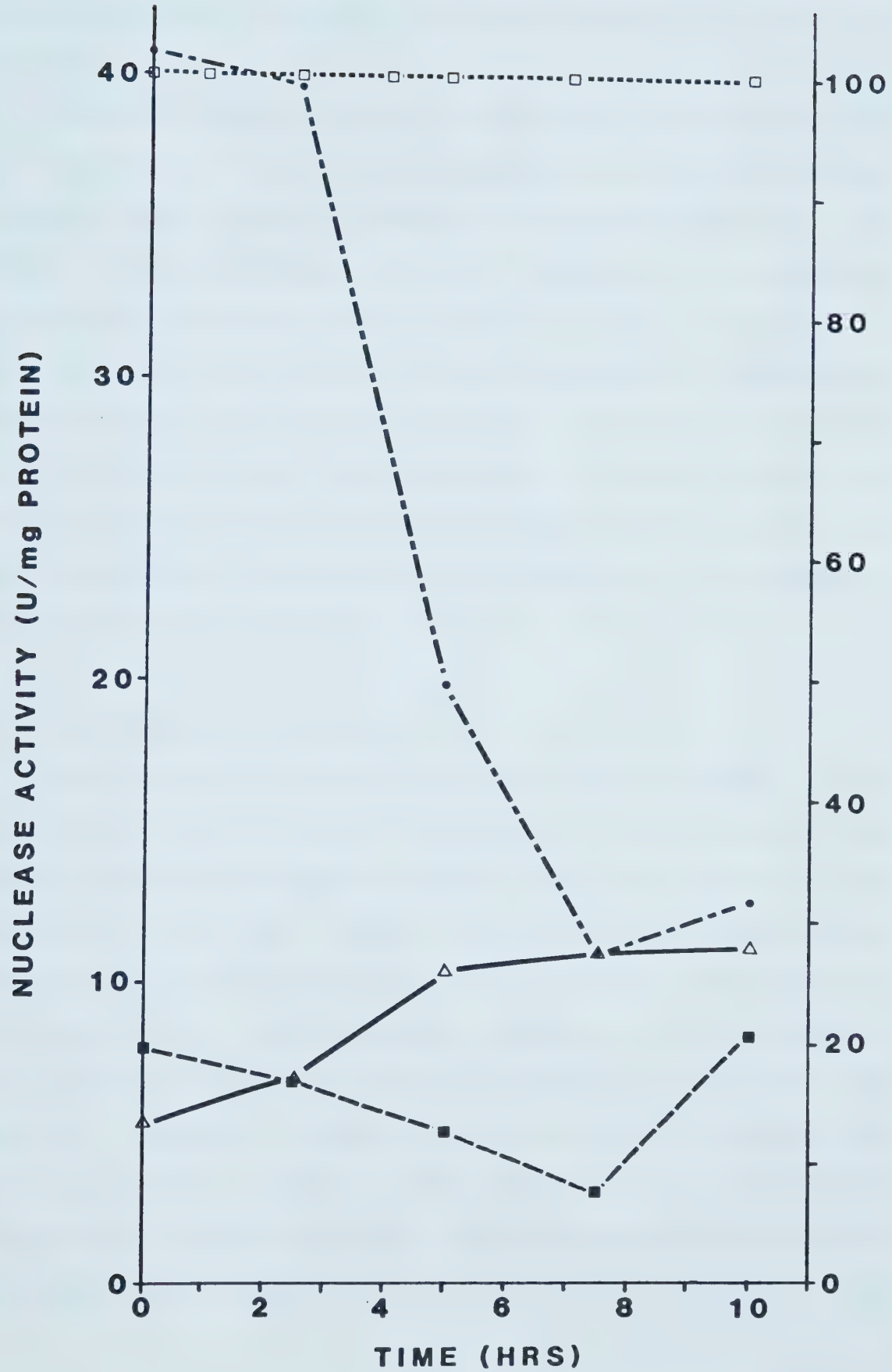
Haploid cells were transfered from sporulation medium to yeast extract-glucose broth to a density of 5×10^7 cells/mL. Cell extracts were obtained at 2.5 hr intervals by passing the cells through a French pressure cell at 103 MPa. The nucleases were isolated and assayed as described in the methods section.

Mg^{2+}-independent Endonuclease ($\times 10^2$): •••••••

Mg^{2+}-dependent Endonuclease ($\times 10^2$): △——△

Mg^{2+}-dependent 5'-exonuclease ($\times 10^4$): ■——■

% Vegetative Cells: □·····□


NUCLEASE ACTIVITY (U/mg PROTEIN)
% VEGETATIVE CELLS
TIME (HRS)
0
10
20
30
40
0
20
40
60
80
100
0
2
4
6
8
10

to the changes seen in the germinating spores, showing an overall increase in activity from relatively low levels at zero time.

The activities of the Mg^{2+}-independent endonuclease and Mg^{2+}-dependent 5'-exonuclease however, were relatively high at zero time in this cell population and decreased over the course of the experiment. The Mg^{2+}-dependent 5'-exonuclease at zero time showed a similar activity to that observed in the haploid cells at 10 hrs under sporulation conditions. This indicates that between 10 hrs in sporulation medium and the time the cells were transfered to growth medium, the activity of this enzyme remained fairly constant, unlike the Mg^{2+}-independent endonuclease which continued to increase in activity during this time.

Nuclease Stability *in vivo*

Variations in nuclease activities may be brought about by changes in the rates of synthesis or degradation of the enzyme, inhibition or activation by some other means, or by a combination of these. To decide between these possibilities, it was desirable to establish the stability of the nucleic acid-degrading enzymes *in vivo*. This was determined by inhibiting protein synthesis and measuring the rate of decrease in enzyme activity. Protein synthesis was inhibited under growth, sporulation and germination conditions by cycloheximide. To determine the concentration of cycloheximide required to stop protein synthesis, the

inhibition of ^{14}C-leu incorporation into hot acid-precipitable materials was measured as described in the methods section. Concentrations of 0.1, 0.5 and 1.0 mg/mL cycloheximide were tested, all of which stopped the uptake of ^{14}C-leu into proteins by more than 95%. To ensure complete inhibition of protein synthesis under the various culture conditions, a concentration of 0.5 mg/mL was used in all subsequent inhibition experiments.

The *in vivo* stability of the nucleic acid-degrading enzymes in exponential phase cells growing on yeast extract-glucose broth is shown in Fig 13. The three nucleases were all quite stable, decreasing in activity only very slowly under these conditions. Therefore, although the Mg^{2+}-dependent 5'-exonuclease activity varied somewhat, none of the enzymes were synthesized or degraded to any great extent in cells inhibited with cycloheximide.

In both the haploid and diploid strains under sporulation conditions, there was very little change in the activities of the nucleic acid degrading enzymes when protein synthesis was inhibited by cycloheximide (data not shown). This suggests that under these conditions, the nucleases were very stable over the duration of the experiment and the rate of proteolysis of these enzymes was slow.

The stability of the nucleic acid-degrading enzymes in both strains under germination conditions was determined as well (data not shown). The nucleases were, in general, very

FIGURE 13

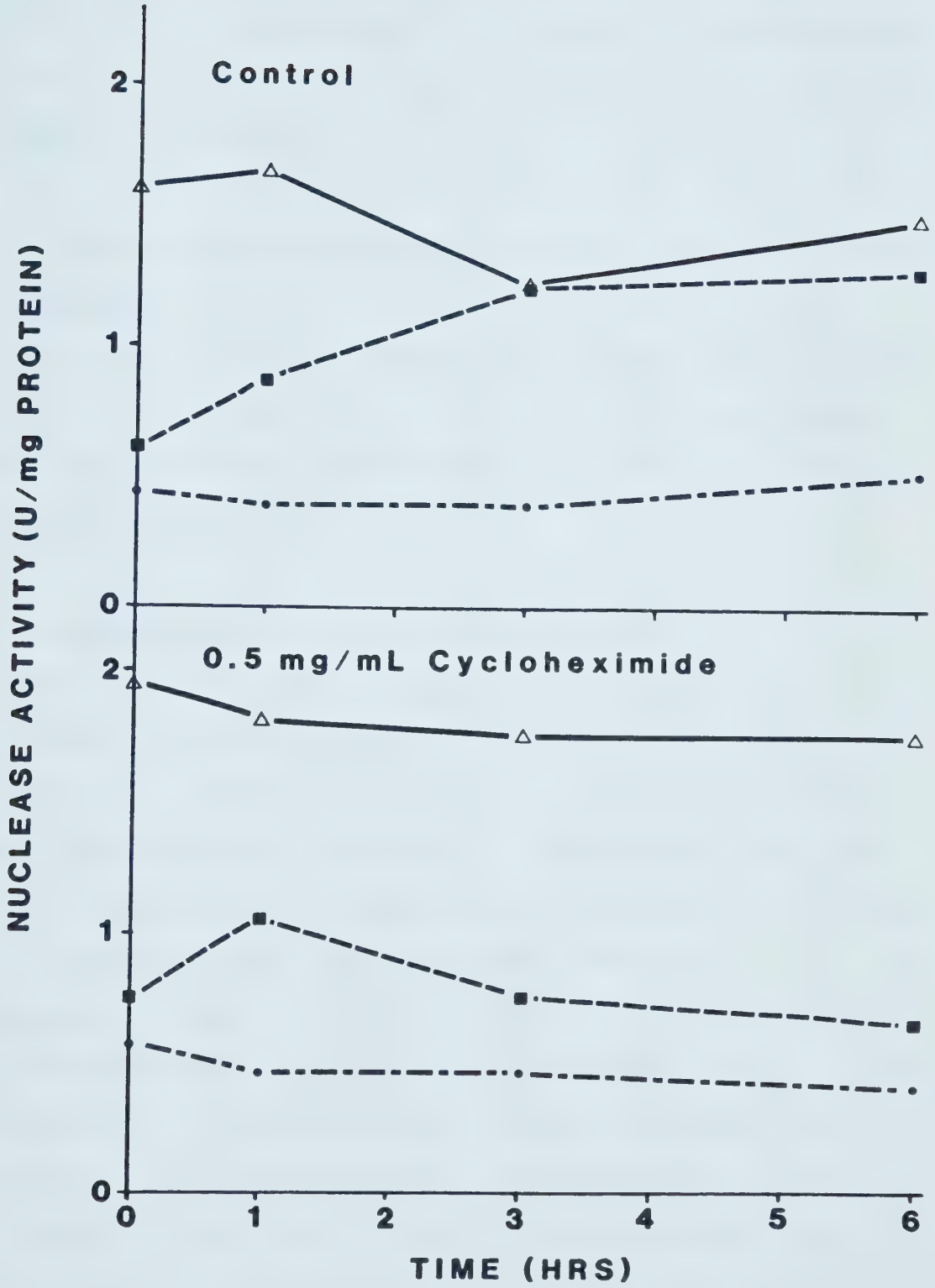
In vivo Stability of Nucleic Acid-Degrading Enzymes

Cells were grown to exponential phase in yeast extract-glucose broth. Cycloheximide was added to a final concentration of 0.5 mg/mL in the test culture. The cells were harvested at the indicated times and broken by passing them through the French pressure cell at 103 MPa. The nucleases were isolated and assayed as described in the methods section.

Mg^{2+}-independent Endonuclease ($x10^2$): •—-—•

Mg^{2+}-dependent Endonuclease ($x10^2$): △——△

Mg^{2+}-dependent 5'-exonuclease ($x10^4$): ■·—·■


Control
0.5 mg/mL Cycloheximide
NUCLEASE ACTIVITY (U/mg PROTEIN)
TIME (HRS)
0
1
2
3
4
5
6

stable under these conditions. One exception was the Mg^{2+}-independent endonuclease in the haploid strain. The rapid decrease in the specific activity of this enzyme as seen in fig 12 was substantially reduced by cycloheximide. Therefore, this decrease was probably due to *de novo* synthesis of proteases.

E. Electrophoretic Characterization of the Mg^{2+}-independent Endonuclease

The Mg^{2+}-independent endonuclease showed the greatest variability throughout the cell cycle and it was decided to investigate its electrophoretic mobility and other physical and chemical characteristics.

Mg^{2+}-independent Endonuclease Activity Stain

To determine the electrophoretic behavior of the Mg^{2+}-independent endonuclease on polyacrylamide gels it was essential to develop an activity stain for this enzyme. Several methods were attempted. The first method was that of Blank and Dekker (1975). After electrophoresis of the enzyme on a non-denaturing polyacrylamide slab gel, it was incubated overnight with poly(A) which diffused into the gel and was digested by the nuclease. Staining the gel with Toluidine blue O, which binds to oligonucleotides, turns the background blue with clear areas indicating the position of the enzyme. Using this method, long streaks rather than sharp bands were obtained with a very light background.

Bands may have become apparent if the incubation time was shortened but this would be limited by the rate at which the substrate diffused into the gels, so this method was abandoned.

The next method which was attempted involved incorporating poly(A) into the gel matrix along with spermine tetrahydrochloride (Karpetsky *et al*, 1980). It was found that the protein migrated only a short distance into the gel even after prolonged electrophoresis, which was probably due to the presence of poly(A), spermine or both. In addition, the poly(A) was not fixed in the gel matrix, possibly because the oligonucleotides were too short to remain immobile.

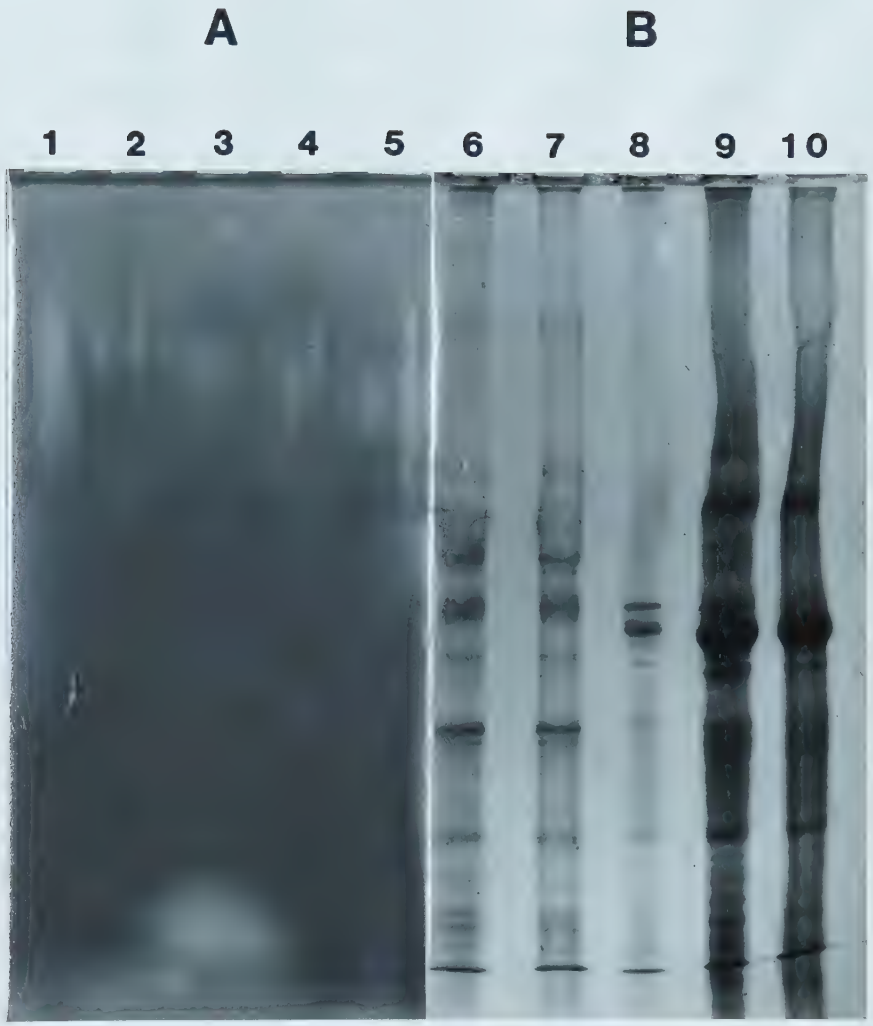
This method was modified by replacing the spermine with SDS and adding poly(A) to the upper reservoir buffer so that if the substrate did migrate, it would be continually replaced. Although improvement in the banding of the nuclease was observed in the activity stain, distortions in the lanes were seen in both activity and protein stains, as shown in Plate 3. Therefore, this method was abandoned since the effects of these distortions on enzyme mobility are unknown.

The method which was adopted involved the use of an agarose gel containing poly(A) over which the polyacrylamide gel was laid, as suggested by Dr. K. L. Roy and described in the methods section. A typical activity stain from a polyacrylamide gel loaded with increasing amounts of

PLATE 3

Substrate-Containing Polyacrylamide Gel Electrophoresis of Mg^{2+}-independent Endonuclease

A 10% polyacrylamide gel was cast containing 0.1% SDS and 0.1 mg/mL Poly(A). Electrophoresis was carried out at a constant current of 12 mA through the stacking gel and 30 mA through the separating gel for a total of 7 hrs. When electrophoresis was complete, the gel was cut in half and after extraction of SDS, section A was stained for nuclease activity and section B was stained for protein. Tracks 1 and 6 and tracks 2 and 7 each contain 6.5×10^{-3} units of Mg^{2+}-independent endonuclease activity isolated from exponential phase cells and treated at 100°C for 2 min with 1% SDS and 1%SDS, 8 *M* urea, respectively. Tracks 3 and 8 contain 20×10^{-3} units of partially purified nuclease isolated from stationary phase cells and treated with 1% SDS at 100°C for 2 min. Tracks 4 and 9 and tracks 5 and 10 each contain 50×10^{-3} units of nuclease isolated from stationary phase cells and treated at 100°C for 2 min with 1% SDS and 1% SDS, 8 *M* urea, respectively.


A
B
1 2 3 4 5 6 7 8 9 10

nuclease is shown in Plate 4. Some diffusion of the enzyme is apparent but a greater degree of banding was seen here than in the previous methods. Since the polyacrylamide gel did not contain poly(A), interference of protein migration or degradation of the substrate during electrophoresis would not occur. In addition, the extent to which the activity is developed could be easily controlled by varying the incubation time of the gels before staining, which was usually about 6 hrs. Therefore, this method was used for all further work in this area.

Nuclease Characteristics on Polyacrylamide Gels

The Mg^{2+}-independent endonuclease has been found to be very stable in SDS, as shown in Plate 5. It could be activated even after a 15 min exposure to 1% SDS in a boiling water bath, but activity was irreversibly lost when the enzyme was treated with β-mercaptoethanol indicating that disulfide bonds are required either at the active site, or to maintain protein conformation, or both. This sensitivity to β-mercaptoethanol was evident when the enzyme was detected by the activity stain or by the standard assay. Therefore, the loss of activity seen in plate 5, lane 5 probably was not just due to the electrophoretic separation of subunits required for activity.

Plate 6 represents an activity stain of a polyacrylamide gel loaded with Mg^{2+}-independent endonuclease prepared from exponential phase (lanes 1 and 3) and

PLATE 4

Activity Stain of a 10% Polyacrylamide Gel in SDS of Increasing Amounts of Mg^{2+}-independent Endonuclease

Electrophoresis of the Mg^{2+}-independent endonuclease was carried out for 12 hrs as described in the methods section. The nuclease was isolated from stationary phase cells and partially purified by heparin agarose chromatography. Tracks 1 through 5 contain 2.5×10^{-3}, 7.5×10^{-3}, 12.5×10^{-3}, 25×10^{-3} and 50×10^{-3} units of enzyme activity, respectively.

1
2
3
4
5

PLATE 5

Activity Stain of a 10% Polyacrylamide Gel in SDS of Mg^{2+}-independent Endonuclease Heated for Increasing Times

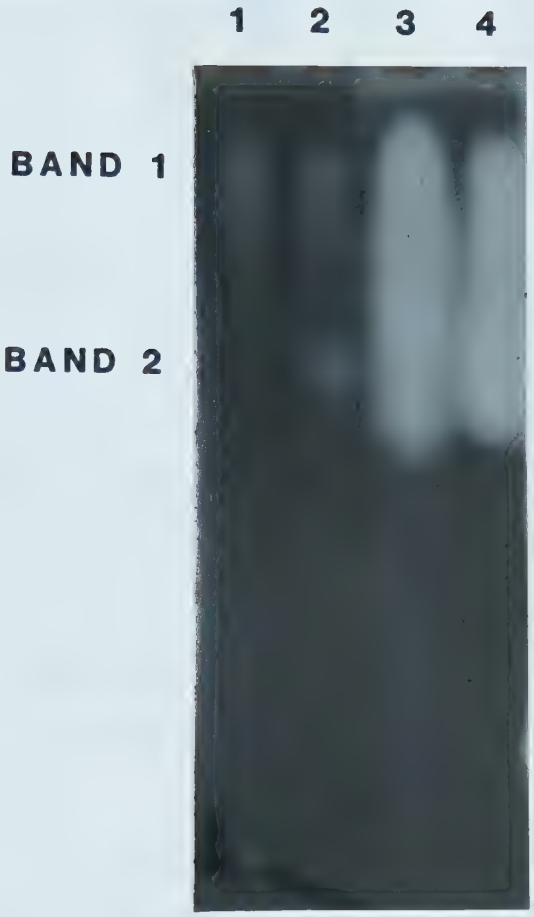
Electrophoresis of the Mg^{2+}-independent endonuclease was carried out for 8 hrs as described in the methods section. The enzyme was isolated from stationary phase cells and partially purified by heparin agarose chromatography. All tracks contain 0.05 units of enzyme activity. The enzyme applied to tracks 1 through 4 was heated in 1% SDS at 100°C for 2, 4, 8 and 15 min, respectively. Lane 5 contains nuclease heated in 1% SDS, 1% β-mercaptoethanol at 100°C for 2 min.

1
2
3
4
5

PLATE 6

Activity Stain of a 10% Polyacrylamide Gel in SDS of Exponential and Stationary Phase Mg^{2+}-independent Endonuclease Developed for Increasing Times

Electrophoresis of the Mg^{2+}-independent endonuclease was carried out for 8 hrs as described in the methods section. Tracks 1 and 3 each contain 0.01 units of enzyme isolated from exponential phase cells and heated in 1% SDS at 80°C for 10 min. Tracks 2 and 4 each contain 0.05 units of enzyme isolated from stationary phase cells and heated in SDS as above. Tracks 1 and 2 were incubated at 37°C for 4 hrs while tracks 3 and 4 were incubated overnight before staining.


1
2
3
4
BAND 1
BAND 2

stationary phase (lanes 2 and 4) cells. Two bands of activity were observed in lane 2 but only the slower migrating band (band 1) appeared in lane 1. However, the presence of the faster migrating band (band 2) was revealed upon overexposure of the activity stain, as seen in lane 3. The presence of two bands may be due to a separate nuclease which increases in activity as the cells enter stationary phase. However, band 2 may also be a breakdown product of band 1.

Electrophoresis of the Mg^{2+}-independent endonuclease with molecular weight markers was performed to determine the size of the two bands of activity and the results are presented in Plate 7. Band 2 has a molecular weight of about 50,000-68,000 d, while band 1 is greater than 116,000 d. A third band of activity is observed in some cases running slightly faster than band 2. This band may be an artifact of electrophoresis, a separate nuclease activity or a modified form of band 2.

Since there was such a large difference in the apparent molecular weights of the slow and fast migrating bands, their separation by gel filtration and ion exchange chromatography was attempted. Fig 14 shows the profile of a Sephadex G-100 superfine column eluted in the presence of SDS. While there was some separation of the protein components in the sample, a single peak of Mg^{2+}-independent endonuclease activity was obtained at the void volume of the column. Chromatography of proteins on a column of

PLATE 7

Molecular Weight Determination of Mg^{2+}-independent Endonuclease on 7.5% Polyacrylamide Gel in SDS

Electrophoresis of the Mg^{2+}-independent endonuclease was carried out for about 3 hrs as described in the methods section. The enzyme was isolated and purified from stationary phase cells and 17 units were loaded onto lane 2 after treatment with 1% SDS at 100°C for 2 min. The molecular weight standards in track 1 are as follows: a. β-galactosidase, b. phosphorylase B, c. bovine serum albumin, d. γ-globulin heavy-chain, e. ovalbumin, f. γ-globulin light-cnain and g. RNase A. The standards were heated at 100°C for 10 min in 1% SDS and 1% β-mercaptoethanol before electrophoresis.

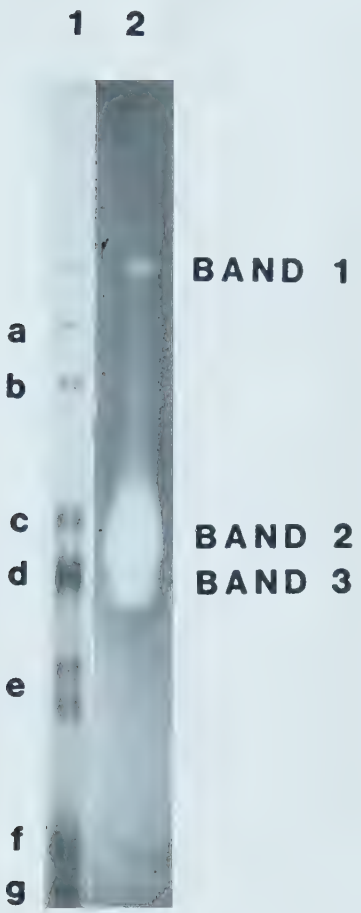

1 2
BAND 1
a
b
c
BAND 2
d
BAND 3
e
f
g

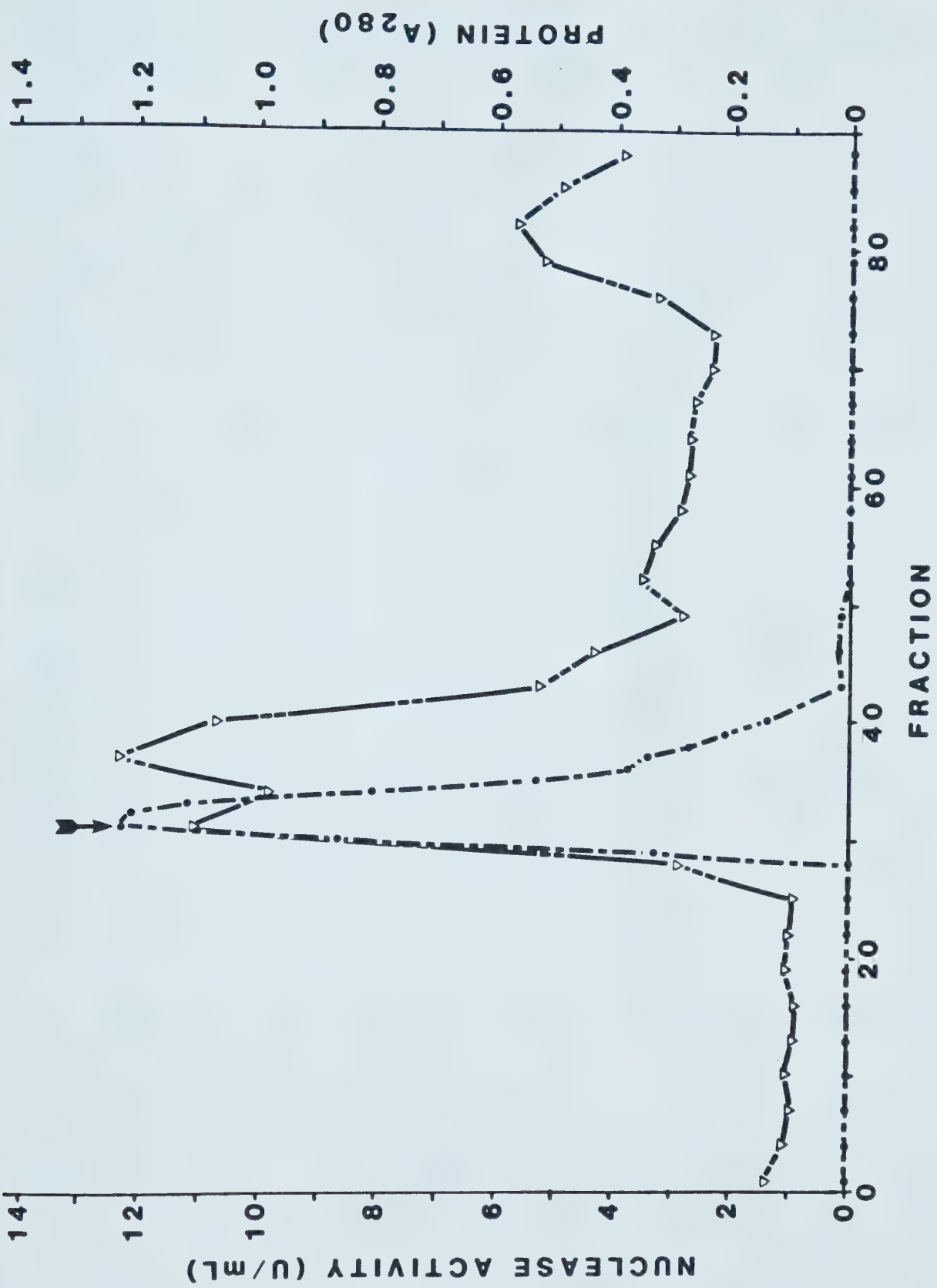
FIGURE 14

Sephadex G-100 Superfine Column Chromatography of Mg^{2+}-independent Endonuclease

The Mg^{2+}-independent endonuclease was isolated from stationary phase cells as described in the methods section. The sample was heated in a boiling water bath in the presence of 1% SDS for 2 min. Three mL (about 4 units of enzyme activity) was applied to a 2.5x43 cm column of Sephadex G-100 superfine equilibrated with 10 m*M* Tris-Cl, 0.1 m*M* NaCl, 0.1% SDS, pH 7.5. Fractions of 2.5 mL were collected at a flow rate of 0.2 mL/min at room temperature. The fractions were diluted 25-fold in 0.1 *M* Tris-Cl, 1% Triton X-100, pH 7.0 and assayed for 2 hrs for Mg^{2+}-independent endonuclease activity. The arrow indicates the position of elution of Blue Dextran 2000.

Mg^{2+}-independent Endonuclease (x10^2): •••—••

Protein: ▽———▽


NUCLEASE ACTIVITY (U/mL)
PROTEIN (A_{280})
FRACTION
0
2
4
6
8
10
12
14
0.2
0.4
0.6
0.8
1.0
1.2
1.4
20
40
60
80

DEAE-trisacryl in the presence of SDS should separate polypeptides of different size since they would bind different amounts of SDS and the larger the molecule, the greater its negative charge would be. Chromatography of the Mg^{2+}-independent endonuclease revealed a single peak of enzyme activity. Therefore, since the enzymes could not be separated into two components by other means, perhaps the slower migrating band is an aggregate of the components of the faster migrating bands or the presence of multiple bands is an artifact of electrophoresis.

Another possibility is that the presence of multiple bands was the result of proteolysis of band 1. To determine if this was the case the Mg^{2+}-independent endonuclease was isolated from stationary phase cells in the absence and presence of the protease inhibitors PMSF and benzamidine-HCl. These samples were run on a polyacrylamide gel and the activity stain is shown in Plate 8. There is no difference in the migration pattern of the enzyme isolated in the absence or presence of the inhibitors.

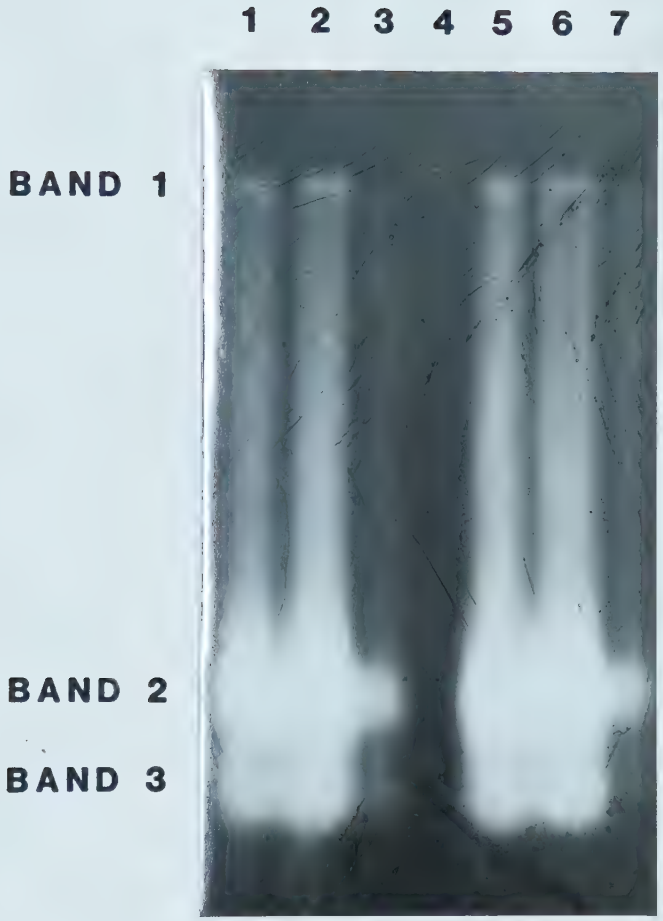
F. Physical and Chemical Characterization of the Mg^{2+}-independent Endonuclease

Some of the physical and chemical properties of the Mg^{2+}-independent endonuclease were determined so that this enzyme could be compared to those reported in the literature.

PLATE 8

Activity Stain of a 7.5% Polyacrylamide Gel in SDS of Mg^{2+}-independent Endonuclease Isolated in the Absence and Presence of Protease Inhibitors

Electrophoresis of the Mg^{2+}-independent endonuclease was carried out for 5 hrs as described in the methods section. Tracks 1-3 contain 27×10^{-3}, 15×10^{-3}, and 1.6×10^{-3} units, respectively, of enzyme isolated from stationary phase cells in the absence of protease inhibitors. Tracks 5-7 contain 19×10^{-3}, 13×10^{-3} and 1.7^{-3} units, respectively of enzyme isolated from stationary phase cells in the presence of 10^{-3} *M* PMSF and 10^{-3} *M* benzamidine-HCl.


1 2 3 4 5 6 7
BAND 1
BAND 2
BAND 3

The substrate specificity of the Mg^{2+}-independent endonuclease is shown in Table 8. The enzyme is more active with poly(C) than poly(A), which may indicate a preference of the enzyme for pyrimidine nucleotide bonds. It degrades dsDNA or ssDNA at a very low rate if at all.

The effects of various divalent metal ions on the activity of the Mg^{2+}-independent endonuclease were determined as well. The assays were carried out in the presence of either 10 m*M* EDTA, Mg^{2+}, Cu^{2+} or Fe^{2+}. No effect on the activity of the enzyme compared to the EDTA control was observed.

The pH optimum curves of the Mg^{2+}-independent endonuclease isolated from exponential and stationary phase cells are shown in Fig 15. Although there are some differences in the profiles obtained, the similarities observed suggest that the pH optimum for both populations is between 6.5 and 7.0. The enzyme isolated from stationary phase cells is somewhat more active at acidic pH values than the nuclease isolated from exponential phase cells.

The thermal stability of the Mg^{2+}-independent endonuclease was determined in the absence and presence of SDS as shown in Fig 16. If SDS was added, the enzyme was diluted 25-fold into 0.1 *M* Tris-Cl, 1% Triton X-100, pH 7.0 before being assayed. In the absence of SDS, the enzyme was stable up to 55°C for 10 min and lost activity quickly at higher temperatures. In the presence of SDS, the activity could be recovered even after heating to 95°C for 10 min.

TABLE 8

Substrate Specificity of the
Mg^{2+}-independent Endonuclease

Substrate	Nuclease Activity (U/mL) ($x10^1$)	% Activity
Poly(A)	9.93	100
Poly(C)	13.85	139.4
RNA	4.72	47.5
ssDNA	0.02	0.2
dsDNA	0.03	0.3

FIGURE 15

Optimum pH of the Mg^{2+}-independent Endonuclease

Mg^{2+}-independent endonuclease assays were carried out to determine the enzyme activities at the pH values indicated. The enzyme preparations used were isolated from exponential and stationary phase cells and purified by heparin agarose chromatography.

Enzyme Isolated from Exponential Phase Cells: O·—--O

Enzyme Isolated from Stationary Phase Cells: ▲——▲

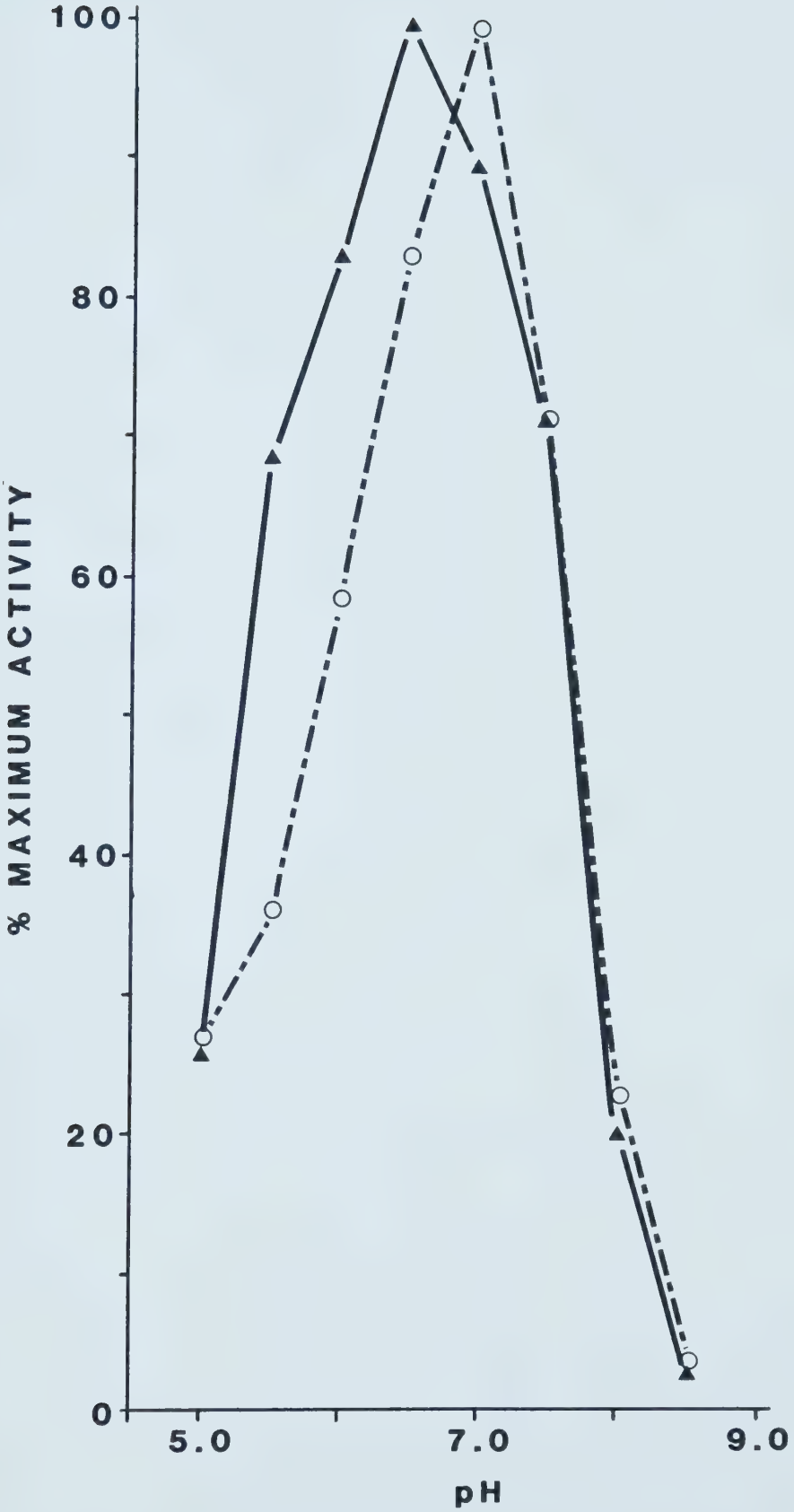

100
80
60
40
20
0
% MAXIMUM ACTIVITY
5.0
7.0
9.0
pH

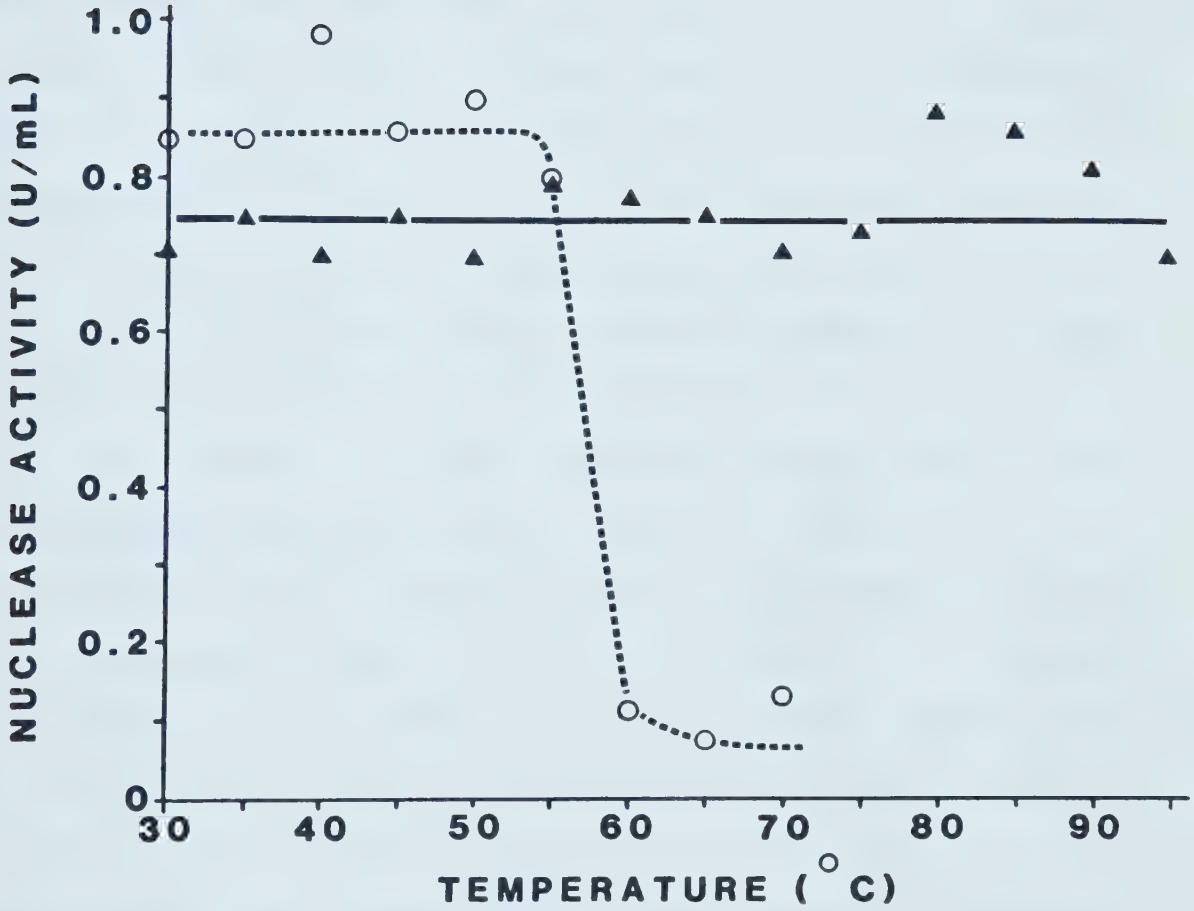
FIGURE 16

Temperature Stability of the Mg^{2+}-independent Endonuclease

The Mg^{2+}-independent endonuclease was heated for 10 min at the indicated temperatures in the absence and presence of 0.1% SDS. The activities of the nuclease heated in the absence of SDS were determined by the standard assay. The enzyme heated in the presence of SDS were diluted 25-fold into 0.1 *M* Tris-Cl, 1% Triton X-100, pH 7.0 and assayed for 60 min.

Nuclease Heated Without SDS: O·····O

Nuclease Heated With SDS: ▲——▲


NUCLEASE ACTIVITY (U/mL)
1.0
0.8
0.6
0.4
0.2
0
30
40
50
60
70
80
90
TEMPERATURE (°C)

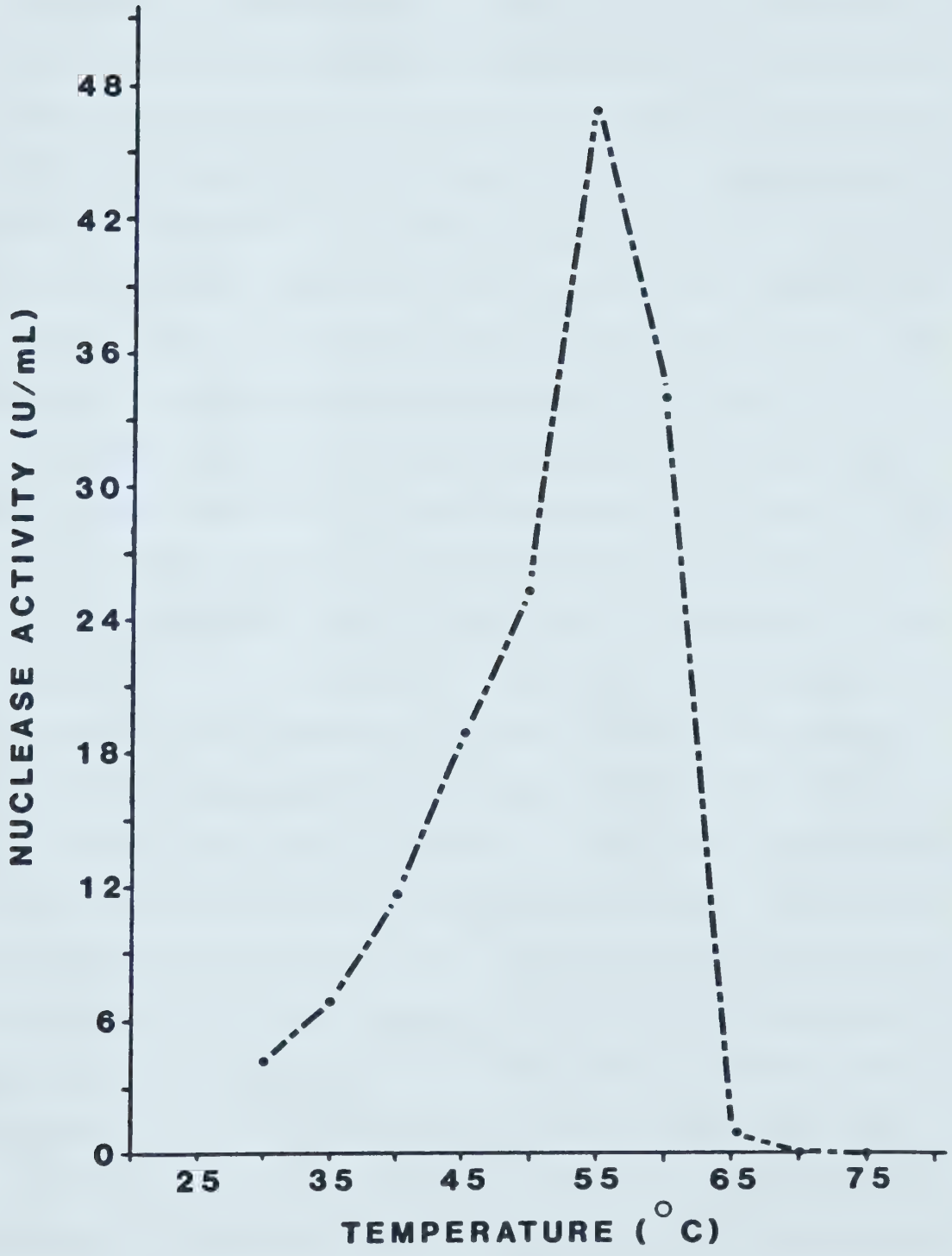
The optimum temperature of the Mg^{2+}-independent endonuclease was determined by standard assay at the indicated temperatures and the results are presented in Fig 17. It is most active at 55°C when assayed for 30 min, with the activity decreasing rapidly on either side of this temperature.

The degradation products of the Mg^{2+}-independent endonuclease were determined as described in the methods section. DEAE-cellulose chromatography of the degradation products revealed the presence of five major peaks which eluted between 20 m*M* and 0.5 *M* NH_4HCO_3 when the absorbance of the fractions at 260 nm was measured. The peak fractions were treated with snake venom phosphodiesterase I and alkali as described in the methods section and then spotted onto 57 cm strips of #40 Whatman filter paper. After chromatography the spots were visualized under short wave ultraviolet light. Chromatography of the products treated with phosphodiesterase I revealed three spots which migrated with adenosine, 5'-AMP and, presumably, ADP phosphorylated at the 3' and 5' positions. Visualization of the degradation products treated with alkali revealed 2'-AMP and 3'-AMP exclusively. These results show that the Mg^{2+}-independent endonuclease yielded 3'-phosphate terminated short oligonucleotides as end products.

FIGURE 17

Optimum Temperature of the Mg^{2+}-independent Endonuclease

Standard Mg^{2+}-independent endonuclease assays were carried out at the temperatures indicated. The enzyme was isolated and purified by heparin agarose chromatography as described in the methods section.


NUCLEASE ACTIVITY (U/mL)
48
42
36
30
24
18
12
6
0
25
35
45
55
65
75
TEMPERATURE (°C)

IV. DISCUSSION

Three nucleic acid-degrading enzymes from *Saccharomyces cerevisiae* identified as a Mg^{2+}-independent endonuclease, a Mg^{2+}-dependent endonuclease and a Mg^{2+}-dependent 5'-exonuclease were investigated. The changes in activities of these enzymes were followed throughout the life cycle of this organism in an effort to clarify their intracellular functions. Initially it was necessary to establish techniques to obtain efficient cell breakage and conditions to assay the nucleases specifically and carry out synchronous sporulation and germination of the cells.

To verify that the nuclease assays were specific enough to avoid cross-reactivity, it was necessary to isolate and purify the enzymes. Exposure of the cell extracts to 2 *M* KCl and centrifugation at 100,000xg separated the Mg^{2+}-independent endonuclease and the Mg^{2+}-dependent 5'-exonuclease, which were both found in the supernatant, from the Mg^{2+}-dependent endonuclease found in the pellet. The nucleases from each fraction were then partially purified by chromatography on heparin agarose. In this way it was discovered that the Mg^{2+}-independent endonuclease cross-reacted in the assay for the Mg^{2+}-dependent 5'-exonuclease. However, it was also found that Zwittergent 3-14 inhibited the 5'-exonuclease completely. This detergent had no effect on the Mg^{2+}-independent endonuclease. Therefore, the activity of the 5'-exonuclease was estimated by subtracting the activity in the presence of

the detergent from the total activity obtained in its absence.

Heparin agarose chromatography of the solubilized cell extract 100,000xg pellet showed that only the Mg^{2+}-dependent endonuclease was present in any significant amounts. This indicates that centrifugation at 100,000xg in the presence of 2 *M* KCl is an effective means of separating the Mg^{2+}-independent endonuclease and Mg^{2+}-dependent 5'-exonuclease activities from the Mg^{2+}-dependent endonuclease. Since little or no activity was observed when the column fractions were assayed for the 5'-exonuclease, the Mg^{2+}-dependent endonuclease did not cross-react in the assay for the Mg^{2+}-dependent 5'-exonuclease. This is likely due, to a large extent, to the fact that the assay for the 5'-exonuclease is carried out at pH 8.0 at which the Mg^{2+}-dependent endonuclease is very inactive (von Tigerstrom, 1982). In addition, assays for the Mg^{2+}-dependent 5'-exonuclease were carried out in the absence of Zwittergent 3-14, which is required to activate the Mg^{2+}-dependent endonuclease. Since the activity of the 5'-exonuclease is so low, cross-reactivity of this enzyme in the assay for the Mg^{2+}-dependent endonuclease would not significantly contribute in the determination of the Mg^{2+}-dependent endonuclease activity. Furthermore, the assay for the Mg^{2+}-dependent endonuclease was carried out in the presence of Zwittergent 3-14, which inhibited the Mg^{2+}-dependent 5'-exonuclease.

The transition from exponential- to stationary phase, sporulation and germination are the periods in the life cycle which show the greatest change in nucleic acid metabolism. Therefore, these stages were examined for variations in the activities of the nucleic acid-degrading enzymes.

Sporulation was carried out at a cell density of $5x10^7$ cells/mL. It was desirable to carry out sporulation at the highest cell density possible in order to obtain sufficient amounts of the three nucleases for assay. Above $5x10^7$ cells/mL a considerable delay in the onset of ascospore formation was observed. Sporulation in yeast is a highly aerobic process (Esposito and Klapholz, 1981), so this delay may be due to limitations in the available oxygen in the medium at high cell densities.

Cell extracts were prepared by breaking whole cells in the French pressure cell since it could be carried out rapidly and at low temperatures while sphaeroplast formation required extended incubations at 30°C under non-growing conditions. However, with the French pressure cell, mature spores could not be broken. Treating the spores with Zymolyase 60,000 would probably not have had a significant effect since the outer layer of the spore wall is made up of chitin, which is resistant to β-glucanases (Ballou *et al*, 1977). The inability to break spores was a major drawback since it resulted in a gap in the determination of the nuclease activities during the life cycle. As will be

discussed shortly, the nuclease activities in spores could be deduced to some extent from the results.

The Mg^{2+}-independent endonuclease showed the greatest change throughout the life cycle of *S. cerevisiae*. It increased almost 4-fold as the cells entered stationary phase and more than 6-fold during sporulation. The similarities in the variation of the specific activity of the enzyme isolated from the haploid and diploid strains under sporulation conditions indicates that the changes were not sporulation specific, but reflect the general conditions of nutritional deprivation.

The only differences in the Mg^{2+}-independent endonuclease activity between the haploid and diploid strains were observed when the cells were transfered from sporulation to germination conditions. In the mature spores, the RNA content is relatively low (Croes, 1966). Therefore, the Mg^{2+}-independent endonuclease would no longer be required and was degraded or inactivated in some way as the spores matured, decreasing in activity considerably as seen at zero time in germination.

In the haploid strain, the activity of the Mg^{2+}-independent endonuclease was maintained at high levels, as seen at zero time in germination, showing that it was still needed in these cells. Unlike the diploid strain, which enters a semi-dormant state upon spore maturation, the haploid strain remains vegetative. These cells continually degrade and re-synthesize their macromolecules (Clare and

Oliver, 1979) which would account for the maintenance of high levels of the Mg^{2+}-independent endonuclease under these conditions.

From these data, the physiological role of the Mg^{2+}-independent endonuclease may be deduced. The activity of this enzyme increased considerably at times in the life cycle when the total RNA content of the cells decreases as much as 50% (Croes, 1966). The majority of the RNA which is degraded is made up of rRNA (Polakis and Bartley, 1966) and the extent of increase in the Mg^{2+}-independent endonuclease activity suggests a role in the degradation of rRNA. Whether it is also involved in the degradation of mRNA or tRNA needs to be determined. It may also play a role in the destabilization of mRNA by removing the poly(A) tails, since it is very active with this substrate. It is doubtful that it is involved in the processing of RNA transcripts since it is not associated with the nucleus. However, it may degrade the discarded portions of the RNA primary transcripts after they are excised.

The suggestion that the Mg^{2+}-independent endonuclease is involved solely in RNA metabolism is supported by the substrate specificity of the enzyme. Although it is less active with high molecular weight RNA as substrate than with poly(A) and poly(C), it has little or no activity with native or heat-denatured DNA. This fact as well as its intracellular location indicates that it is not involved in DNA metabolism *in vivo*. The difference in activity of the

enzyme with the homopolymers as compared with native RNA may reflect differences in the structure of the substrates, rather than an inability of the nuclease to degrade them.

The Mg^{2+}-independent endonuclease has been found to be very stable *in vivo* under most conditions, losing activity only very slowly when protein synthesis was inhibited by cycloheximide. The exception to this occurred when the haploid cells were transfered from sporulation to germination conditions. In the presence of cycloheximide, the activity of the Mg^{2+}-independent endonuclease decreased about 40%, which is probably due to proteolytic degradation of the enzyme. However, in the absence of the antibiotic, the rate of decrease in the activity of this enzyme doubled, which may be due to the synthesis of additional protease, a specific enzyme inhibitor, or both.

The increase in Mg^{2+}-independent endonuclease activity during stationary phase and sporulation was also abolished by cycloheximide. Therefore, these increases were probably due to *de novo* synthesis of the enzyme, rather than to an existing but inactive enzyme.

The changes in activity of the Mg^{2+}-dependent 5'-exonuclease throughout the life cycle of *S. cerevisiae* were very similar, although not as pronounced in most cases, as the variations of the Mg^{2+}-independent endonuclease. A 2-fold increase in activity was seen as the cells entered stationary phase, while increases of about 3.5- and 7-fold were seen in the haploid and diploid strains, respectively,

under sporulation conditions. It is difficult to determine if this difference in the two strains during sporulation is a sporulation specific event since the same general trend was observed with both strains. This suggests that, like the Mg^{2+}-independent endonuclease, the increases in activity were a general response by the cells to conditions of nutrient deprivation.

Like the Mg^{2+}-independent endonuclease, the Mg^{2+}-dependent 5'-exonuclease activity decreased considerably as the spores matured, while it was maintained at relatively high levels in the haploid strain under the same conditions. Since the variations in activity of these two nucleases throughout the life cycle of *S. cerevisiae* appear to be so similar, the 5'-exonuclease may also be involved in RNA metabolism *in vivo*. This is supported by Stevens (1978) observation that this enzyme has little or no activity with DNA as substrate. Stevens (1980) proposed that the Mg^{2+}-dependent 5'-exonuclease functions in the degradation of messenger RNA since it hydrolyses the substrate in a 5'-->3' direction. This is supported by the results of this investigation since the activity of the 5'-exonuclease increased during the life cycle at times when the rates of overall RNA degradation increase. The generally low specific activity of this enzyme indicates that its role in RNA digestion is more restricted than that of the Mg^{2+}-independent endonuclease, likely to the degradation of mRNA. It is possible that the Mg^{2+}-independent endonuclease

generates uncapped 5'-ends for the 5'-exonuclease, but this might not be a major factor in RNA metabolism since the Mg^{2+}-independent endonuclease creates 5'-OH ends, which are resistant to degradation by the Mg^{2+}-dependent 5'-exonuclease. The nuclease described by Belhadj *et al*, (1982) does create 5'-phosphate ends so this enzyme may have such a role *in vivo*.

Inhibition of protein synthesis by cycloheximide showed that the Mg^{2+}-dependent 5'-exonuclease was also quite stable *in vivo*. The increase in its specific activity when the cells entered stationary phase or when they sporulated was abolished by this inhibitor indicating that these increases were likely due to *de novo* synthesis of the enzyme. Similarly, the decrease in specific activity during outgrowth was reduced by cycloheximide, suggesting that this decrease was due to *de novo* synthesis of proteases, a specific inhibitor, or both.

The variations in activity of the Mg^{2+}-dependent endonuclease throughout the life cycle of *S. cerevisiae* were almost exactly opposite to the changes observed with the other two nucleases. The specific activity decreased when the cells entered stationary phase and when both the haploid and diploid strains were placed under sporulation conditions. However, up to a 2-fold increase in Mg^{2+}-dependent endonuclease activity was seen in the diploid strain during the first few hours of sporulation. Under the same conditions, this increase was not observed in the

haploid strain, which does not undergo meiosis. When the cells were transfered from conditions of nutrient deprivation to growth medium, the activity of the Mg^{2+}-dependent endonuclease increased.

Several proposals have been advanced concerning the functions of this enzyme. Fraser (1979) and Fraser and Cohen (1983) proposed that a similar nuclease of *N. crassa* is involved in DNA repair. This does not seem likely since 90% of the enzyme is located in vacuoles or bound to the inner mitochondrial membrane, with only 2% associated with the nucleus. The vacuolar location reported by Fraser (1979) and Fraser and Cohen (1983) suggests that the Mg^{2+}-dependent endonuclease of this organism may be involved in RNA metabolism in some way.

Resnick *et al* (submitted for publication) observed a 10- to 20-fold increase in the activity of the Mg^{2+}-dependent endonuclease from *S. cerevisiae* during the first few hours of sporulation. They suggest that it is involved in the high levels of recombination which occur early in meiosis. While my observations are in general agreement with theirs and might seem to support the proposal of Resnick *et al*, the intracellular location of this enzyme argues against it since it is inaccessible to the majority of cellular DNA. However it could act as a recombinase with mitochondrial DNA. The variations in its activity throughout the life cycle suggest that it is not involved in RNA metabolism, but it is quite active with poly(A) and RNA as

substrate (von Tigerstrom, 1982) so such a role cannot be entirely ruled out.

The increase in activity of the Mg^{2+}-dependent endonuclease during germination was abolished by cycloheximide. Cycloheximide inhibits cytoplasmic, but not mitochondrial protein synthesis. This tends to confirm that this enzyme is coded for by nuclear, rather than mitochondrial DNA as suggested by von Tigerstrom (1982) and Fraser and Chow (1983). It also indicates that any increases were due to *de novo* synthesis, while the decreases were probably due to degradation. Otherwise, it is very stable *in vivo*, losing activity only very slowly when cells in all stages of the life cycle were treated with cycloheximide.

Since the Mg^{2+}-independent endonuclease showed the greatest variation throughout the life cycle of *S. cerevisiae*, it was decided to study it in more detail. It was hoped that a determination of its characteristics would help to clarify its function and would allow a comparison of this enzyme with those reported in the literature. It was also important to establish whether the activity was due to one enzyme.

The electrophoretic properties of the Mg^{2+}-independent endonuclease were studied using an activity stain for the enzyme on polyacrylamide gels. Two bands of activity were revealed. The slower migrating component (band 1) has an apparent molecular weight of more than 100,000 d while the faster migrating component (band 2) has a molecular weight

of about 50,000-68,000 d. Band 2 was present in significant amounts only in stationary phase cells while band 1 remained fairly constant in both exponential- and stationary phase cells. These results indicate that a different nuclease is being synthesized as the cells enter stationary phase. This is supported by the optimum pH values of the Mg^{2+}-independent endonuclease isolated from exponential and stationary phase cells. While the activity profiles of both are very similar above pH 7.0, the enzyme isolated from stationary phase cells is more active at acidic pH values, indicating the presence of a form of Mg^{2+}-independent endonuclease which is slightly more stable at acidic pH values.

Attempts to isolate the two apparent forms of the enzyme by gel filtration and ion exchange chromatography, both in the presence of SDS, were not successful. It was expected that molecules of different molecular weights would be separated by chromatography on DEAE-Trisacryl in the presence of SDS since the larger the molecule, the more SDS it would bind and the higher its negative charge would be. However, only one peak of activity was observed in each case, indicating that band 1 may be an aggregate of band 2, possibly due to the low salt concentrations used during electrophoresis. This would also explain the smearing observed between the two bands in the activity stain. Whether molecules with molecular weights of 50,000 d and 100,000 d in the presence of SDS can be separated on

Sephadex G-100 columns remains to be determined.

A third activity band running slightly faster than band 2 was observed in some cases. While this may represent a different nuclease, this is not likely since the third band was not separated consistently and it was not isolated from either band 1 or 2 by column chromatography. While it seems more likely that the presence of the multiple bands is an artifact of electrophoresis, the presence of a second, similar Mg^{2+}-independent endonuclease activity cannot be ruled out.

The presence of the multiple bands is probably not due to proteolysis of the enzyme since isolation of the nuclease in the presence of protease inhibitors had no effect on the migration patterns on polyacrylamide gels. It is possible that a protease which is resistant to the inhibitors was responsible for this pattern, but the elution profile of the enzyme on column chromatography argues against this.

The Mg^{2+}-independent endonuclease is very stable at 55°C, but it loses activity rapidly and irreversibly at 60°C. However, when SDS was added to the sample before heating, full activity could be recovered after heating up to 95 °C if the SDS was replaced by Triton X-100 before assaying the enzyme (Clarke, 1981). This indicates that SDS protected the enzyme from heat inactivation in some way, possibly by preventing denaturation of the protein at high temperatures. Such a property has not been previously reported for this enzyme. Treatment of the enzyme with

β-mercaptoethanol resulted in an irreversible loss of activity. This indicates that disulfide bonds are required for activity, either at the active site, for conformation of the protein, or both.

The characteristics of the Mg^{2+}-independent endonuclease determined here are very similar to those of the enzymes described by Ohtaka *et al* (1963) and Nakao *et al* (1968). With some variations, they all have similar pH and temperature optima, substrate specificities and produce end products with 3'-phosphate ends. It is different from the enzyme reported by Schulz-Harder *et al* (1979) and Shetty *et al* (1980). While all have similar substrate specificities, end products and temperature optima, the enzymes described in these investigations have a much lower pH optimum. In addition, the enzyme described by Shetty *et al* is inhibited by Cu^{2+} and Fe^{2+}, which had no effect on the nuclease described here. Belhadj *et al* (1982) also reported a Mg^{2+}-independent endonuclease, but it is different from the enzyme described here since it produces 5'-phosphate terminated oligonucleotides as end products.

The specific *in vivo* functions of these nucleases may be further clarified by isolating mutants which are defective in their synthesis and observing the effects on nucleic acid metabolism. Further work is also necessary to determine if the multiple bands on polyacrylamide gels represent separate nuclease activities or are merely artifacts of electrophoresis. In this way it may be possible

to determine whether a unique enzyme is being synthesized as the cells enter stationary phase or if these bands are due to aggregation of the enzyme. If the latter case is true, it may then be possible to determine if these aggregates have any function *in vivo*.

The data seems to indicate a role for the Mg^{2+}-dependent endonuclease in recombination, but its intracellular location makes it inaccessible to the majority of cellular DNA. A more rigorous localization of the enzyme is required to determine if it is also associated with the nucleus. Isolation of various organelles at different stages in the life cycle may reveal whether the relative proportion of the enzyme associated with the organelles shifts, possibly due to the activation of a precursor which would otherwise not be detected.

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